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13. ABSTRACT ***(Maximum 200 Words)***

Estrogen is mitogenic in most estrogen receptor (ER) positive breast cancers and the use of anti-estrogen like tamoxifen has been quite successful in the treatment of this disease. Although patients initially respond well to anti-estrogens, resistant tumors often develop within 5-10 years of treatment. The purpose of this research is to develop mechanistically distinct therapeutics by directly blocking the interaction of ER with coactivator proteins required for its activity. We have identified conformation-sensing peptide probes that detect different estrogen receptor conformations, and which when introduced into cells abolish ER transcriptional activity in transient transfection assays. These same peptides however do not seem to have an effect on endogenous ER activity. Studies are underway to investigate this discrepancy. Since the androgen receptor (AR) is highly expressed in most breast cancers, it is recognized now that AR may also play a role in the progression of these cancers. We therefore extended our studies to include the investigation of the mechanisms underlying androgen receptor (AR) mediated transcription, using peptides that we identified in the primary ER screen. We found that the ligand binding domain of AR has a unique structure which prohibits its interaction with the p160 coactivators, and that it recruits coactivator protein in a manner which is distinct from other nuclear receptors. It is anticipated that exploitation of the complexities of ER and AR action will lead to the development of novel breast cancer therapeutics.

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Introduction:

The objective of this research project is to identify novel therapeutic strategies for breast cancer treatment by targeting estrogen receptor (ER) function in cancer cells. The estrogen receptor is a transcription factor whose activity is modulated by the nature of the bound ligand. The classical pharmacology models postulated that the receptor exists in only two forms, active and inactive. Accordingly, binding of an agonist switches the receptor from an inactive state to an active one, and that antagonists work simply by competitively blocking agonist access to the receptor. The discovery of the selective estrogen receptor modulators (SERMs), which function as either receptor agonists or antagonists in a tissue selective manner, challenged the classical model and highlighted the complexity of ER action. A more complex model which takes into account the differential interaction of ER-ligand complexes with cellular cofactor proteins has emerged. It has become clear in recent years that protein-protein interactions govern many biological processes, including transcriptional activation by ER, and that ligand induced structural alterations in ER can influence its interaction with cofactor proteins. We proposed to identify small peptides with which to detect the conformations of various ER-ligand complexes. We expect that these peptides will be useful for the analysis of the complex ER pharmacology. Additionally, we believe that peptides which detect important protein-protein interaction surfaces on ER will be discovered which will highlight new targets for drug development in breast cancer.

Body:

In the previous two granting periods we applied a combinatorial peptide screening approach and identified peptides that detect ligand-induced structural alterations within the estrogen receptor (ER), some of which recognize protein-protein interaction surfaces within the receptor (1, 2). Specifically, several peptides interact with the p160 coactivator binding pocket within ER, and when overexpressed in cells these peptides blocked the association of ER with coactivators, leading to disruption of ER-mediated transcription activation. We have also demonstrated that the mechanisms by which tamoxifen and estradiol manifest agonist activity are not the same. We found that the ER-tamoxifen and ER-estradiol complexes may recruit distinct coactivator proteins, suggesting ER activity can be differentially regulated by targeting different receptor:cofactor interactions. Additionally, high affinity ERβ-specific peptides were identified which, when introduced into cells, specifically disrupt the activity of ERβ, but not the activity of the closely related ERα protein (3). This finding was significant since there is no known ER-subtype specific ligands available to dissect the contributions of these two receptor subtypes in estrogen meidiated biological activities. Several novel ER-interacting cofactor proteins were identified in a yeast two-hybrid screen that may facilitate the elucidation of ER pharmacology, and can be used to validate receptor:cofactor interaction surfaces as targets for therapeutic intervention.

The major task in this last granting period is to validate whether targeting the protein-protein interaction surfaces on ER can be used to develop novel therapeutics for tamoxifen-resistant tumors.

(1) Endogenous and transfected ER respond differently to AF-2 targeting peptides

We demonstrated previously that in cells transfected with exogenous ER, co-expression of ER activation function-2 (AF-2) binding peptides efficiently inhibits ER transcriptional activity (2). These peptides contain the signature LxxLL motif (L: leucine, x: any amino acids), a motif found in all p160 coactivators and required for these coactivators to interact with the AF-2 of most nuclear receptors, including ER. We needed to verify whether this class of peptides can also inhibit endogenous ER function before developing these peptides as therapeutics. To test this, we transfected human breast cancer MCF-7 cells with an estrogen-responsive reporter gene and increasing amounts of plasmids expressing the aforementioned peptides. To our surprise we found that although the 2xF6 and GRIP peptides efficiently inhibited ERα transcriptional activity in HeLa cells expressing exogenous ERα (Figure 1A), these same peptides have minimal effect on endogenous ER activity when tested in MCF-7 cells (Figure 1B). This finding was rather confusing, since we have previously used several approaches, including mammalian two-hybrid analysis, GST-pull down assay, and fluorescence co-localization, and have demonstrated that these LxxLL containing peptides interact with ER in vitro and in cells. Based on these findings, we concluded, therefore, that the inhibitory effect of these peptides on ER transcriptional activity is likely due to their ability to compete with p160 coactivator proteins for binding to the AF-2 pocket. Since we failed to show an inhibition of these peptides on endogenous ER activity, we wanted to re-confirm that these peptides indeed interact with ER with a more direct measurement of their interaction inside of cells. We utilized the fluorescence polarization assay to address this question. We fused the ERα with the red fluorescence protein

(RFP) and the peptides with the cyan fluorescence protein (CFP). The fluorescence energy transfer can only occur when the RFP and GFP are brought to close proximity. Using this analysis, we confirmed that the exogenously transfected ER indeed interacts with LxxLL peptides in living cells (Figure 2 and (4)). This assay, however, will not be able to address whether the LxxLL peptides interact with endogenous ER. The result of this work and other findings using the fluorescence resonance energy transfer (FRET) assay is published in *Molecular Endocrinology 16 (3): 487-496, 2002.*

To rule out the possibility that transfection may not be able to deliver sufficient amounts of peptides into cells to disrupt endogenous ER:cofactor interactions, Dr. Ganesan in this lab has generated an adenovirus expressing the Gal4DBD-2xF6 peptide fusion protein. When she infected MCF-7 cells with these viruses, she found again that the 2xF6 peptide was able to inhibit the activity of the exogenously transfected ERα, but not the activity of the endogenous ERα. Not all the receptors we tested show such discrepancy between the endogenous and transfected proteins. Dr. Kimbrel in this lab had identified a peptide, Lx 23, which interacts with the AF-2 of the progesterone and glucocorticoid receptors (PR and GR). She was able to show that the Lx23 peptide can efficiently inhibit both PR and GR activities in cells expressing these endogenous receptors (5). Therefore, we believe it is possible to develop novel therapeutics by targeting the AF-2 pocket of nuclear receptors (such as PR and GR); however, the pharmacology of ER appears to be more complex than we originally anticipated.

The fact that the peptide could not inhibit endogenous ER activity in MCF-7 cells is quite intriguing. We first considered that the affinity of these peptides may not be high enough to disrupt endogenous receptor:cofactor interactions. This is unlikely since the peptide efficiently disrupts the interaction between cofactors and the exogenously expressed ER which has the same protein sequence as the transfected receptor. Additionally, the affinity of the one copy F6 peptide is approximately 100 nM (6), similar to the affinity of SRC1 LxxLL motif for ER. We expected that the affinity of the two-copy peptide 2xF6 should be much higher than the single copy one (as shown in the previous report and (2)). We concluded, therefore, that affinity alone can not explain why these peptides do not inhibit endogenous ER activity.

We next thought it is possible that the endogenously expressed receptor may be modified in a way that distinguishes it from the transfected ER, so that its transcriptional activity does not require the recruitment of p160 coactivators through the AF-2 region. However, it has been demonstrated by chromotin immunoprecipitation (CHIP) analysis that the endogenous ER expressed in MCF-7 cells indeed recruits the p160 coactivators such as SRC-1, GRIP1, and AIB-1 to the target gene promoter in an estradiol-dependent manner. Therefore, introduction of the LxxLL containing peptides in cells should affect ER activity, unless the recruitment of p160s to endogenous ER does not require the AF-2, or that the p160 associated ER on the target gene promoters accounts for only a small fraction of the total pool of transcriptionally active ER. Since ERα has two activation function domains, AF-1 and AF-2, we also consider it possible that the AF-1 may be the predominate activation function utilized by endogenous ER to recruit coactivators in MCF-7 cells, and that the transfected ER may have overwhelmed the endogenous system. Instead of engaging the AF-1 coactivators, therefore, it utilizes the more abundant p160s to manifest activity. If that's the case it would explain why only the exogenously-transfected ER is subjected to the inhibition by AF-2 binding peptides. This is supported by the observation that

in HepG2 cells, which allows ER to manifest more AF-1 activity, the LxxLL-containing peptides were not as efficient an antagonist as they were in the AF-2 dominant HeLa cells (2). Additionally, we also found that the activity of the androgen receptor (AR), which is considered to have minimum contribution from the AF-2, is minimally affected by the expression of AF-2 binding peptides (see below).

There is, however, additional evidence suggesting that the transcription and translation of proteins inside cells may be coordinated by RNA-binding proteins (7). It has been proposed that RNA binding proteins may coordinate the localization of transcripts that encode proteins which may function in the same pathways much like the operon in prokaryotic cells. It is possible that the transcripts of endogenous ER and cofactor proteins may be clustered together and associated with one another when translated; therefore, exogenously-expressed peptides do not have a change to interact with endogenous ER. As a first step to distinguish between these mechanisms, we are currently working with the CHIP assay to determine whether the expressed peptides are associated with endogenous ER on the target gene promoter. If the expressed peptides are indeed associated with ER on the target gene, it would suggest that the AF-2 may not be required for endogenous ER activity. If the peptides do not associate with ER on the target gene, it would suggest that the intracellular organization of proteins or perhaps other mechanisms may have precluded these peptides from interacting with the endogenous ER.

(2) The activity of the androgen receptor is regulated in a manner distinct from other nuclear receptors

The androgen receptor (AR), another structurally related steroid hormone receptor, is widely expressed in breast cancer cells, however the role of AR in breast cancer is still controversial. Some studies suggest that the transcriptionally active AR may play a protective role in the progression of breast cancer, while others have observed a correlation of AR expression with the invasiveness of breast tumors. Studies have shown that medroxyprogesterone acetate (MPA), a progestin which is frequently used as second line hormonal therapy for the treatment of metastatic breast cancer, may manifest its activity through binding and activating AR in breast tumors. Therefore, in addition to ER and PR, AR may also be a target for therapeutic intervention in breast cancer. We feel that the study of AR pharmacology will help the analysis of the roles of these steroid hormone receptors in the progression of this disease.

In the previous report, we described the discovery of two peptides that bind not only to estradiol-activated ER but also to the agonist activated AR (and PR). These two peptides, D30 and D11, contain the signature LxxLL motif and bind to the AF-2 of AR in a strictly agonist-dependent manner. Although the AF-2 of most nuclear receptors by itself can recruit coactivators required for transcriptional activity, the AF-2 of AR appears to have minimal transcriptional activity on its own. This could be due to the unique conformation of the AR AF-2. We found that although AR shares a high degree of homology with other steroid hormone receptors, it was not able to interact with the LxxLL motifs that most nuclear receptors interacted with, including the p160 coactivators SRC-1 and GRIP-1 (Figure 3). In fact it appears that the dominant activation function in AR is the AF-1, because the AF-1 alone is just as active as the agonist activated full length AR. Because our peptides bind to the AF-2 of AR and their binding

appears to correlate well with the transcriptional activity of the receptor, we utilized these peptides to determine the role of AF-2 in AR mediated transcription. When over-expressed in cells, both the single copy and the two-copy D30 peptide have only minimal effects on AR transcriptional activity, while under the same conditions these peptides completely abolished PR activity. We concluded from this result that the AF-2 of AR is likely not involved in the recruitment of coactivators.

It has been demonstrated previously that the amino- and carboxyl-termini of AR can interact with each other (8-10). He et al has recently discovered a FXXLF motif (F: phenylalanine, X: any amino acids, L: leucine) located in the amino terminus of AR, which appears to mediate the interaction between the amino- and carboxyl-termini of AR (11). Since the FxxLF motif bears similarity with the LxxLL motif, we wanted to determine if the LxxLL motif we discovered functiond similarly to the FxxLF motif. It was proposed that the purpose of the AR N-/C- interaction is to lock the ligand in its ligand binding pocket, as the ligand binding domain (LBD) of the AR when expressed alone has a high ligand off-rate (12). However, when the AR LBD was co-expressed with the amino terminus of AR, the ligand off-rate could be partially restored. We found that co-expression of our D30 peptide could achieve the same effect as the amino terminus of AR (Figure 5). We thus believe that when bound by an agonist, the AF-2 of AR must undergo a conformational change, a change which is recognized by our D30 peptide as well as the amino terminus of the receptor. Interaction between the amino- and the carboxyl-termini of AR in turn stabilizes the ligand binding within the ligand binding pocket, allowing AR to efficiently activate transcription.

In addition to mimicking the AR amino terminus FxxLF motif, we believe the D30 peptides may also recognize a conformation on the AF-2 of AR which was not revealed by the N-/C- interaction analysis. Specifically, we observed that the conformational changes induced by RU486, a weak agonist of AR, could be detected in a mammalian two-hybrid assay using the D30 peptide and the full length AR (Figure 6B). This interaction correlated with the weak partial agonist activity of RU 486 on AR-mediated reporter gene expression (Figure 6A). The N-/C- interaction, although able to predict the agonist activity of many AR ligands, failed to detect the partial agonist activity of RU486 (Figure 6C). Furthermore, while RU486 was not able to support the N-/C-interaction, it efficiently induced the expression of an androgen responsive MMTV-Luc reporter gene by activating an AR construct containing a VP16 acidic transactivation domain fused to its amino terminus (Figure 6D). Fusion of VP16 to AR circumvents the need to recruit AR-specific coactivators required for its activity, thus serving as an indicator of the ability of a ligand to promote the association between the receptor and specific DNA elements. Since RU486-bound VP16-AR was able to form a complex with DNA that is stable enough to recruit VP16 associated cofactor proteins to the target gene, we believe that the weak agonist activity of RU486-bound AR is likely due to the inability of this receptor-ligand complex to recruit AR-specific cofactors. How the AF-2 conformation, which does not seem to be involved in coactivator docking, regulates the recruitment of cofactors by AR remains to be determined. It is possible that the conformational changes in the AR AF-2 may transduce a structural alteration in the AF-1, permitting its interaction with coactivators. Alternatively, the conformation of the AF-2, if not presented correctly, may pose a stereo hindrance to the AF-1 and passively prevents AF-1 from interacting with coactivators. The results of this study are published in *Molecular Endocrinology 16 (4): 647-660, 2002.* On a similar note, we and others have characterized various ER antagonists, all of which bind to the AF-2 of ER. Some of them inhibit both AF-1 and tAF-2 activity, while compounds like tamoxifen inhibit the AF-2 but spare

the AF-1 activity. The mechanisms by which different AF-2 ligands may be able to affect the activity of AF-1 is interesting, and the understanding of these mechanisms may facilitate the development of more effective therapeutics.

Key Research Accomplishments:

July 1999 - June 2000:

1. Identified conformational-sensitive probes for ER.
2. Developed a cell-based assay system to probe ER conformations.
3. Identified different classes of LxxLL, coactivator:receptor interacting motifs.
4. Demonstrated that tamoxifen- and estradiol-induced transcriptional activities are mediated through different mechanisms.
5. Identified peptide antagonists for estradiol-induced ER transcriptional activity.
6. Identified peptide antagonists that can distinguish between ERα and ERβ.
7. Identified peptide antagonists which block tamoxifen partial agonist activity within intact cells.

July 2000 - June 2001:

1. Identified peptides that bind ERβ with high specificity and affinity using the LxxLL focused library.
2. Validated the ERβ-specific peptides as potent inhibitors of ERβ activity.
3. Confirmed the interactions of peptides identified in our screen with ER in living cells and discovered that different coactivator peptides interact with ER with different kinetics.
4. Identified proteins that interact with ERα in a manner distinct from p160 coactivators.
5. Identified one mechanism by which hormone resistance occurs in prostate cancer cells.

July 2001 - June 2002:

1. Confirmed that the peptides identified in our previous screens interact directly with ER in cells using FRET assay.
2. Discovered that endogenous and transfected ER may manifest their activities in different manners.
3. Identified that the structure of AR AF-2 is distinct from other steroid hormone receptors using short peptides identified in our phage display screen.
4. Analyzed the mechanisms by which the androgen receptor manifests transcriptional activity.

Reportable Outcomes:

Manuscripts:

1. Chang, C.-Y., Walther, P., and McDonnell, D.P. Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer. Cancer Research, 61: 8712-8717, 2001.
2. Weatherman R.V., Chang, C.-Y., Clegg, N.J., Caroll, D.C., Day, R.N., Baxter, J.D., McDonnell, D.P., Scanlan, T.S., Schaufele, F. Ligand-selective interactions of estrogen receptor detected in living cells by fluorescence resonance energy transfer. Molecular Endocrinology 16 (3): 487-496, 2002.
3. Chang, C.-Y. and McDonnell, D.P. Evaluation of ligand-dependent changes in androgen receptor structure by peptide probes. Molecular Endocrinology 16 (4): 647-660, 2002.
4. Pathrose P., Barmina, O. Y., Chang, C.-Y., McDonnell, D. P., Shevde, N. K., and Pike, J. W. Inhibition of 1,25-dihydroxyvitamin D3-dependent transcription by synthetic LxxLL peptide antagonists that target the activation domains of the vitamin D and retinoid X receptors. *Journal of Mineral and Bone Research.* In press (2002).

Meeting Abstracts:

1. Keystone Symposia-Nuclear receptors 2002, Snowbird, Utah. April, 2002.
   Chang, C.-Y. and McDonnell, D.P. Evaluation of ligand-dependent changes in androgen receptor structure using peptide probes

Funding Received:

1. Howard Temin Award (CA95094) from National Cancer Institute to Ching-yi Chang (PI) and Donald McDonnell (Mentor)
   The androgen receptor:cofactor interface-a target for new drugs.

Conclusions:

Anti-estrogens play an important role in the treatment of breast cancers. Because estrogen receptor is required for the maintenance of several normal physiological functions, the ideal drug of choice should have anti-estrogenic activity in the breast but at the same time preserve the beneficial effects of estrogen in other tissues. We have developed conformational probes of ER and demonstrated that various protein-protein interaction surfaces on ER are exposed upon binding different ligands. We also showed that ER activities can be differentially targeted by selectively blocking specific receptor:cofactor interactions using these conformational peptide probes in transient transfection assays. The further development of these peptides into therapeutics for breast cancer is hindered by the observation that the endogenous ER and exogenously transfected ER behave differently in cells. The means by which this occurs is not clear, and we are currently investigating several potential mechanisms. Although developing peptide antagonists for ER cannot proceed until we resolve this problem, peptides that antagonize both exogenous and endogenous PR and GR activities have been developed by colleagues in this lab. We believe that the same approach will likely be successful for the development of novel ER antagonists as we resolve the discrepancy between endogenous and exogenous ER. Since the majority of primary human breast cancers express AR, it has been suggested that AR may also play a role in the disease progression, making it a potential target for therapeutic intervention. We used peptides identified in our initial phage display screen to dissect the mechanisms underlying AR pharmacology. We found that although AR shares high sequence homology with other steroid hormone receptors, the conformation of the AF-2 of this receptor is quite different, which prohibits its interaction with common coactivators. Therefore, the majority of the transcriptional activity of this receptor is manifest through recruitment of AF-1 interacting cofactors. These findings have furthered our understanding of AR pharmacology and will enable the identification of novel targeting strategies to modulate AR activities.

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Figure 1

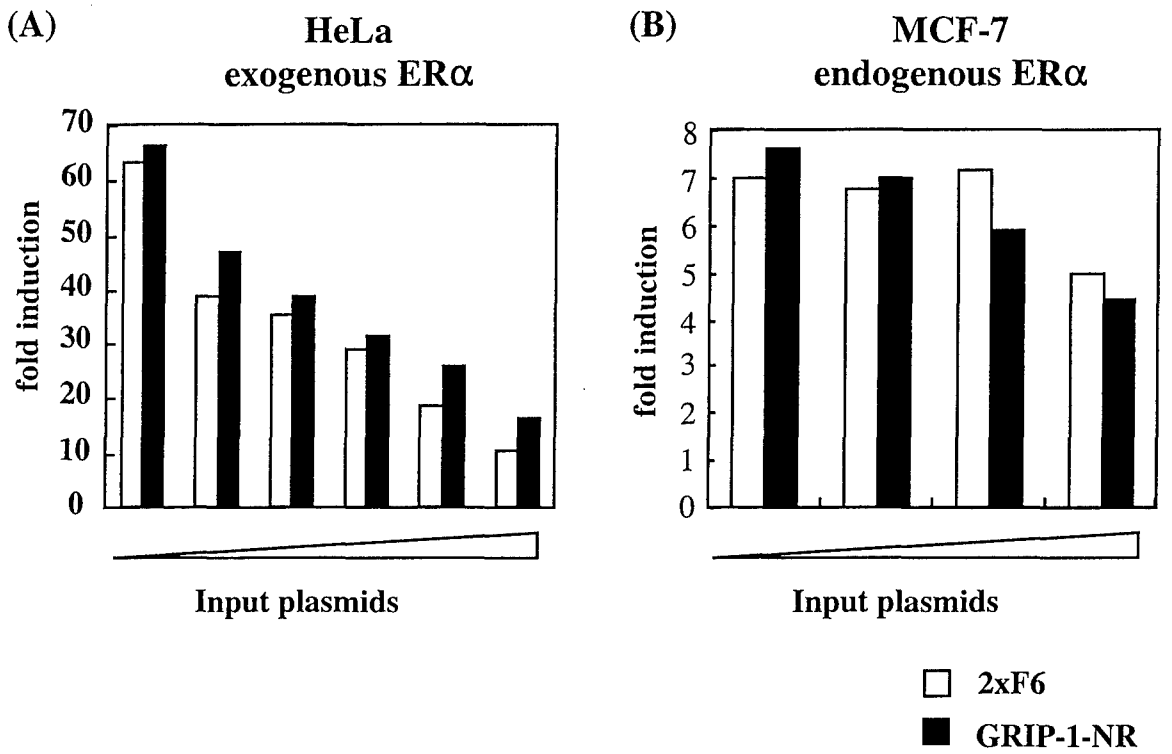


Figure 1. The LXXLL containing peptides efficiently inhibited the activity of a transfected ER, but not the activity of ER expressed endogenously. (A) HeLa cells were transfected with an ERα expression vector, RST7-ERα along with the 3xEREtata-Luc reporter gene and increasing amounts of the Gal4-DBD peptide fusion constructs (either the 2xF6 or GRIP-1 NR box) as indicated. (B) MCF-7 cells which express endogenous ERα were transfected as in (A) but without the ERα expression vector, RST7-ERα. Sixteen hours after transfection, cells were treated with 100 nM 17β-estradiol for an additional 16 h before assaying. Fold induction represents the ratio of estradiol-induced activity versus no-hormone control for each transfection.

Figure 2

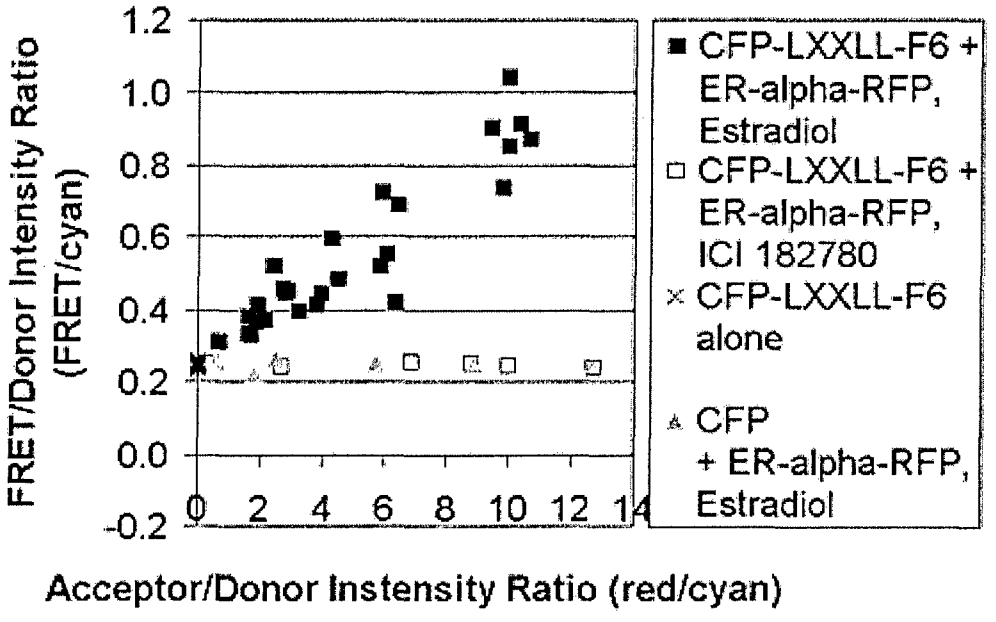


Figure 2. Estrogen-specific interaction of CFP-LXXLL and ERα-RFP in living cells. GHFT1-5 cells were transfected with the ERα-RFP and CFP-LXXLL peptide expression vectors. Transfected cells were grown for 24 h in estrogen-free media. A total of 1 uM of each ligand was added and the cells grown for a further 24 h before data collection. Quantitative fluorescence images were collected with a Hamamatsu ORCA cooled interline camera attached to an Olympus Corp. IX-70 microscope.

Figure 3

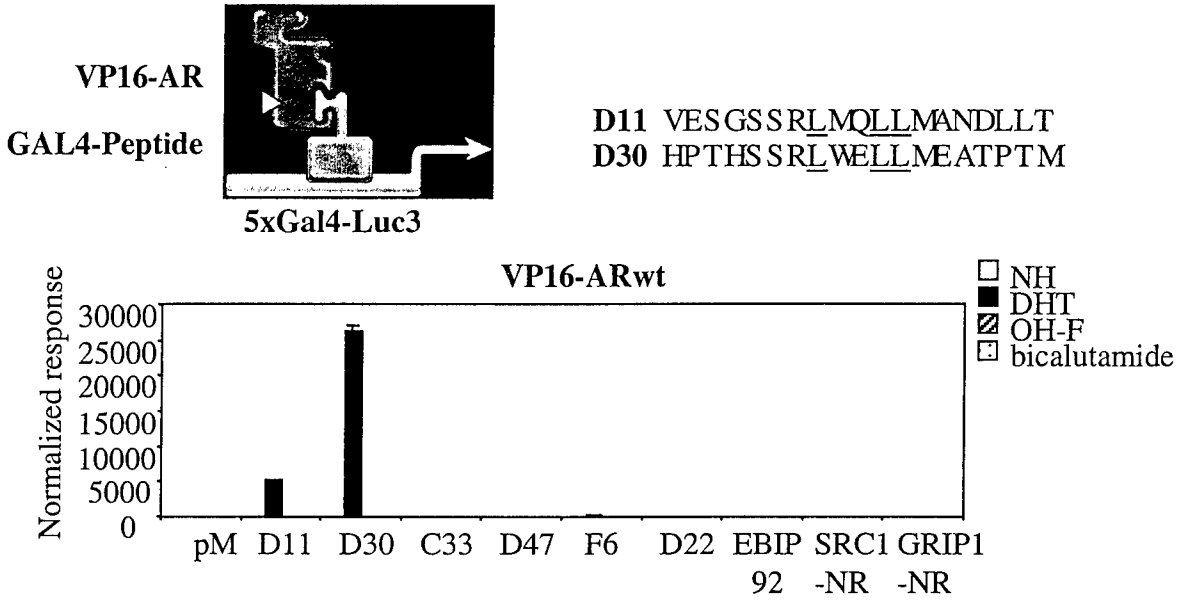


Figure 3. The ligand-binding domain of AR has a preference for LXXLL-peptide binding that is distinct from other nuclear receptors. Different LxxLL-motif containing peptides were fused to the Gal4-DBD, and the full length AR was modified to include a VP16-activation domain at its amino terminus. Interactions between peptides and AR were determined by measuring the expression of a reporter gene containing five copies of the Gal4-response elements. CV-1 cells were transfected with different peptide-Gal4DBD constructs together with either the VP16-AR expression plasmid, and reporter constructs 5xGal4Luc3 and pCMV-βgal. After transfection, cells were treated with either vehicle control (NH), 100 nM 5α-dihydrotestosterone (DHT), 100 nM hydroxyflutamide (OH-F) or 1 μM bicalutamide for 16 h. Luciferase activity was measured and normalized to the activity of the co-expressed β-galactosidase.

Figure 4

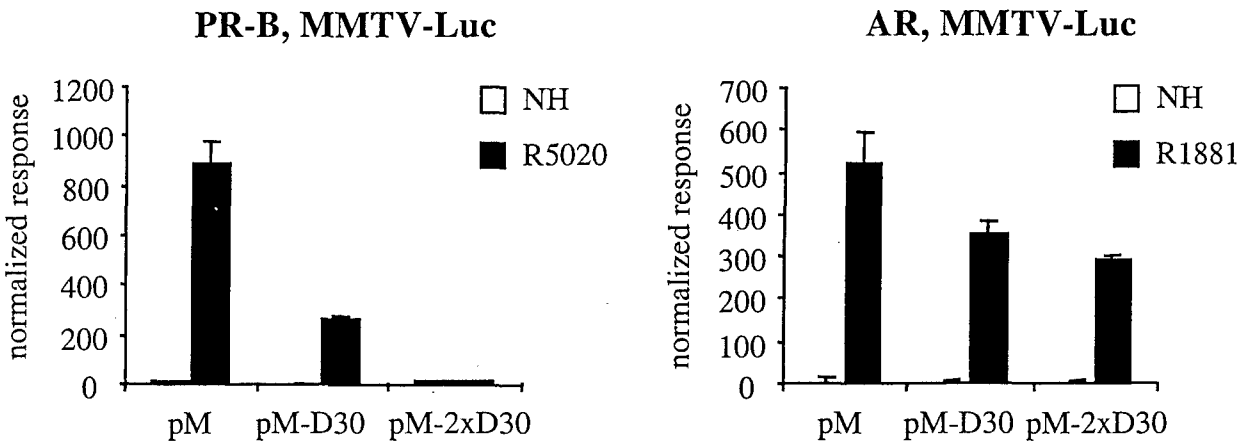
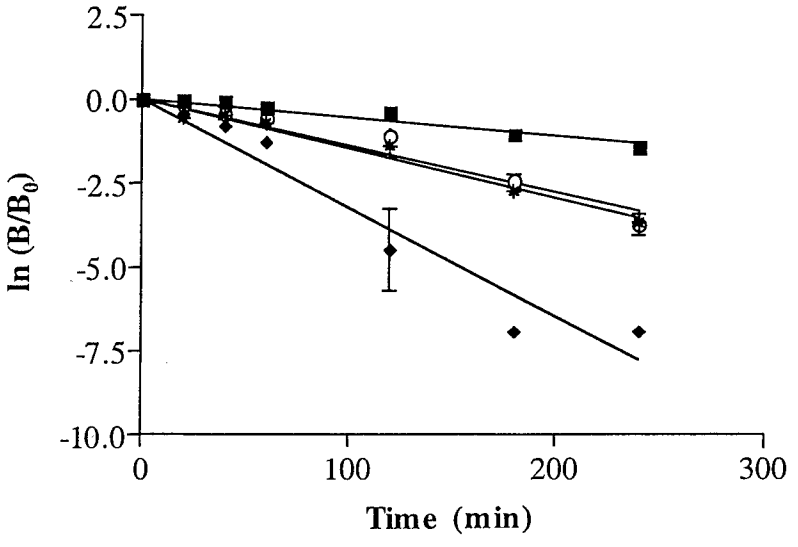


Figure 4. The D30 binding surface on AR does not overlap with that required for coactivator binding. CV-1 cells were transfected with MMTV-Luc, pCMVβgal, and either RS-AR or pKBC-PRB in the presence of either pM, pM-D30 or pM-2xD30 as indicated. After transfection, cells were treated with either vehicle control, the AR agonist R1881 (100 nM) or the PR agonist R5020 (100 nM) for 16 h. Luciferase activity was measured and normalized to the activity of the co-expressed β-galactosidase.

Figure 5



	T 1/2 (min)
■ AR-wt	124.0
* AR507-919 + AR1-501	46.8
◆ AR507-919 + pM	21.4
○ AR507-919 + pM-2xD30	50.0

Figure 5. The D30 peptide functions similarly to the amino terminus of AR in stabilizing ligand binding in the AR-LBD. CV-1 cells were transfected with expression plasmids for either (a) wild-type AR alone, (b) AR507-919 plus AR1-501, (c) AR507-919 plus Gal4DBD (pM) or (d) AR507-919 plus Gal4DBD-2xD30 (pM-2xD30). 24 h after transfection, cells were labeled with 5 nM of ^{3}H-R1881 for 2 h and then a 10,000-fold excess of cold R1881 was added at different time points. Cells were washed 4 times with PBS to remove non-specific binding and then lysed for scintillation counting and protein concentration measurement.

Figure 6

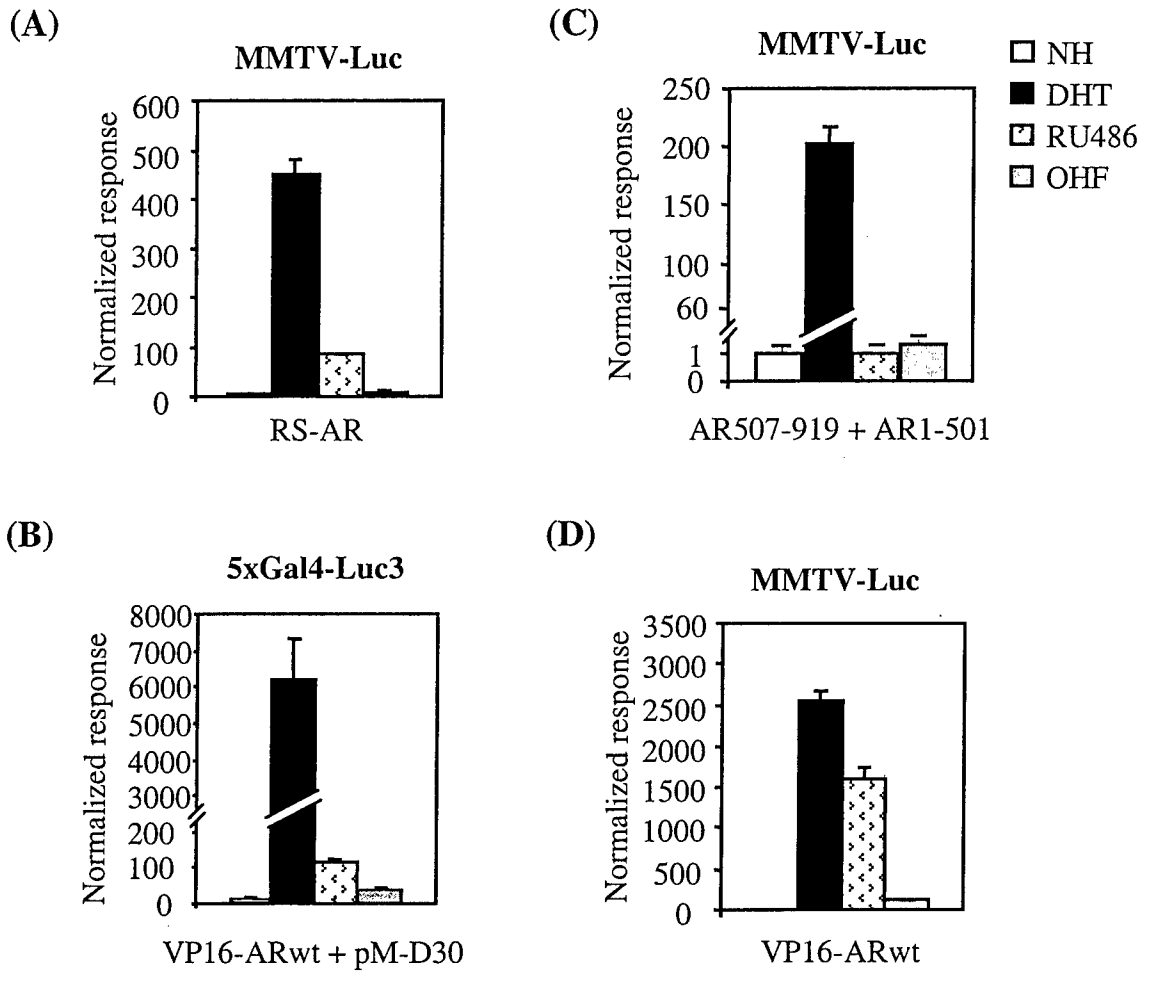


Figure 6. The formation of a D30-binding pocket on AR is required for its transcriptional activity. (A) A reporter gene assay was used to analyze the agonist or antagonist activity of AR ligands. CV-1 cells were transfected with the AR expression plasmid, RS-ARwt together with MMTV-Luc and pCMV-βgal. (B) A mammalian two-hybrid assay was performed to determine the ability of ligand-AR complexes to recruit the D30 peptide. CV-1 cells were transfected with 5xGal4Luc3, pCMV-βgal and pM-30, together with VP16-ARwt. (C) The ability of ligand-AR/LBD complexes to recruit the amino terminus of AR was analyzed. CV-1 cells were transfected with MMTV-Luc, pCMV-βgal and pcDNA-AR1-501, together with pcDNA-AR507-919wt. (D) The ARwt was expressed as fusion proteins to the VP16-acidic activation domain to bypass the need for AR-specific coactivators required for gene transcription, allowing the assessment of the ability of ligands to deliver receptor to DNA. CV-1 cells were transfected with pVP16-ARwt together with MMTV-Luc and pCMV-βgal. After transfection, cells were treated with either vehicle alone, 100 nM DHT, 100 nM OHF or 100 nM RU486 as indicated for 16 h before the luciferase and β-galactosidase activities were determined.

Meeting Abstract

Evaluation of Ligand-dependent changes in androgen receptor structure using peptide probes

Ching-yi Chang and Donald P. McDonnell
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Mutations in the androgen receptor (AR) are frequently found in relapsed prostate cancers, permitting anti-androgens, estrogens, progestins and even glucocorticoids to function as androgens. However, the mechanism by which these mutations enable this shift in AR-pharmacology is still unknown. Resistance to anti-hormone therapy arises also in estrogen receptor (ER)-positive breast cancers, where it is believed that alterations in cofactor expression in the cells permit anti-estrogens like tamoxifen to function as agonists. In support of this hypothesis we have shown that tamoxifen binding to ER allows the presentation of novel protein-protein interaction surfaces on the receptor, enabling it to interact in an ectopic manner with transcriptional coactivators. In this study we wanted to see if the same mechanisms would also apply with respect to anti-androgen resistance. To explore this possibility, we used phage display to identify a series of LXXLL-containing peptides that interact with the AF2 domain of AR. We found that although the binding of peptides to wild-type AR was strictly agonist dependent, these same peptides could also interact with a number of gain of function AR variants containing mutations frequently found in relapsed prostate cancers, in the presence of androgens and non-androgenic activating compounds. This suggests that the agonist activity of these ligands occurs because they, in the background of these mutations, allow AR-AF2 to adopt an active conformation. Initially, this result seems to contradict other findings which suggest that coactivator binding to AR-AF2 is not required for AR activity. In probing this further, we have determined that the role of AR-AF2 appears to be to stabilize the overall structure of the receptor, allowing the amino terminus to interact with appropriate coactivators. This contention is supported by the finding that over-expression of the AF2-binding peptides does indeed block the interaction of the amino- and carboxyl- terminal of AR, but does not attenuate AR transcriptional activity. Thus we believe that mutations in AR which facilitate the formation of an AF2 pocket, have the potential to allow AR antagonists to manifest agonist activity.
Supported by an NIH grant CA-90645 to D.P.M. and a postdoctoral fellowship (DAMD17-99-1-9173) from USAMRAA to C.-Y.C.

[CANCER RESEARCH 61, 8712–8717, December 15, 2001]

Glucocorticoids Manifest Androgenic Activity in a Cell Line Derived from a Metastatic Prostate Cancer[1]

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ABSTRACT

The pathophysiological mechanism(s) by which androgen independence develops in prostate cancer remains to be determined. The identification in many prostate cancer specimens of a mutant androgen receptor, T877A, with altered ligand specificity has provided an explanation for some treatment failures. The T877A mutant androgen receptor recognizes a number of nonandrogenic compounds, including certain estrogens, progestins, and even antiandrogens as androgens. However, a comprehensive screen for hormonal agents which display agonist activity on this mutant has not been performed. In this study, we characterized this clinically important receptor mutant further and found that it can be activated by a wide range of compounds, including a number of endogenous glucocorticoids. Among the most clinically relevant compounds identified are DOC and corticosterone, both of which can effectively activate the mutant receptor at concentrations normally found in blood. Dexamethasone, a synthetic glucocorticoid frequently used in various contexts for prostate cancer therapy, is also recognized as an androgen by the mutant receptor. These unexpected findings suggest the need to: (*a*) reassess the role of adrenally derived glucocorticoids in prostate cancer disease progression; and (*b*) recognize the potential for iatrogenic stimulation of disease progression with certain glucocorticoid interventions.

INTRODUCTION

The most recent estimates published by the American Cancer Society predict 180,000 new cases of prostate cancer annually in the United States and that close to 32,000 men will die of this disease each year (1). Such statistics place this disease second only to lung cancer as the leading cause of mortality in United States males. Although several therapeutic options (radical surgery, external radiotherapy, interstitial brachytherapy) are available for early stage, organ-confined tumors, AA[3] remains the primary, most effective mainstay therapy for advanced disease (2–4). Numerous clinical trials over the past 60 years have demonstrated that suppression of gonadal androgen production by surgical castration or medical intervention (estrogens, GnRH agonists/antagonists) can effectively induce cancer regression for substantial periods of time (reviewed in Refs. 5 and 6). However, progression eventually occurs in most patients treated in this manner. To circumvent this problem, a regimen termed TAA, which combines the use of either castration or GnRH agonists with an antiandrogen such as flutamide, has also been implemented. The effectiveness of TAA for the treatment of prostate cancer, however, remains controversial. Although some well-designed Phase III trials have shown a modest therapeutic advantage, later studies have been unable to confirm these observations (7).

Nevertheless, even while TAA can be very effective initially in patients with advanced disease, an androgen-independent state usually develops within 1–2 years (4, 5), and survival becomes limited beyond that point. The cellular mechanisms leading to the development of androgen-independent tumors are not clear and may involve both AR-dependent and AR-independent pathways. Of particular interest is, however, a syndrome, initially termed the flutamide withdrawal syndrome, which was recognized a decade ago. In ~20–50% of patients who have failed TAA, a paradoxical drop in serum PSA levels (the marker most commonly used to monitor prostate cancer progression) or even improved symptom status was observed after cessation of flutamide (8). The duration of this withdrawal response is variable, typically lasting ~3–6 months. At this point, tumors progress despite castrate levels of androgens.

Different mechanisms have been proposed to explain the development of androgen-independent prostate cancers (reviewed in Ref. 7). Among the best described resistance mechanisms, amplification of AR, which could enhance the impact of residual androgens present in circulation, has been observed in 30% of prostate cancer specimens from patients who have failed AA (9–11). In addition, somatic mutations have been found within AR which alter its pharmacology (12–14). In particular, the T877A mutation (threonine to alanine substitution at amino acid 877), which resides within the ligand-binding domain of AR, has been frequently identified in specimens of hormone refractory metastatic prostate cancer. As a result, an apparent "promiscuous" stimulation of AR-mediated transcriptional activation by estrogens, progestins, and even synthetic antiandrogens such as flutamide has been observed in both the prostate cancer cell line LNCaP, as well as specimens harboring this mutation (15, 16). It is this mutation that is often ascribed as the underlying cause of the flutamide withdrawal syndrome (17), because its incidence is high in flutamide-treated patients and low, though clearly present, in patients who have undergone other treatments (13, 17, 18). Adding to the puzzle, however, is that a significant number of patients whose tumors were subsequently shown to have an 877 AR mutation did not show a clinical flutamide withdrawal response despite discontinuation of the drug (13). Interestingly, Zhao *et al.* (19) has recently shown that the mutations found in MDA PCa 2b cells, a cell line derived from the bone metastasis of an androgen-independent prostate cancer, harbor a double mutation L701H and T877A, which allows the mutant receptor to be activated by cortisol and cortisone; thus, these cells no longer require androgens for growth.

Taken together: (*a*) the observation of the flutamide withdrawal syndrome; (*b*) the identification of mutants of the AR that affect ligand specificity; and (*c*) the ability of patients failing AA monotherapy alone (*e.g.*, castration only) to exhibit frequent modest serological responses to delayed utilization of AR-directed antiandrogens suggest that at this point, such cancers are not strictly androgen independent. Indeed, the identification of receptor mutants permitting altered hormonal specificity suggests that prostate cancer cell growth might be stimulated by other endogenous-circulating hormones at certain stages of cancer progression as a result of this somatic muta-

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[3] The abbreviations used are: AA, androgen ablation; TAA, total androgen ablation; AR, androgen receptor; DPA, diphenylamine; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; DHT, dihydrotestosterone; DOC, 11-deoxycorticosterone; Dex, dexamethasone; GR, glucocorticoid receptor; GnRH, gonadotropin-releasing hormone; OH-F, hydroxyflutamide.

tion. The latter hypothesis is supported by the fact that most flutamide-refractory cancers respond well to bicalutamide, an antiandrogen binding in the ligand-binding domain but with a somewhat different mechanism of action than flutamide (20, 21). Thus, even in flutamide-refractory cancers, it appears as if AR is still somewhat involved in prostate cell growth.

To investigate whether endogenous hormones other than androgens, estrogens, and progestins might contribute to the clinical phenomenon of treatment escape after the emergence of a mutated AR, we decided to test a wide variety of endogenous steroid hormones, as well as synthetic steroids to see if any of these compounds have the ability to activate the AR T877A mutant. It was anticipated that a screen of this nature may lead to the identification of compounds that have the potential to function as AR agonists in certain circumstances. In addition, it may also provide an explanation for the observation that AR-dependent growth of some prostate cancers can occur in the absence of detectable androgens.

MATERIALS AND METHODS

Reagents and Plasmids. All of the chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), except for the bicalutamide, which was a generous gift from Nobex Corp. (Research Triangle Park, NC). The RS-AR and VP16-AR were gifts from K. Marschke (Ligand Pharmaceuticals, San Diego, CA). The RS-AR/T877A and VP16-AR/T877A containing the T877A AR mutant were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The 5xGal4Luc3, MMTV-Luc, and pM-D30 plasmids were described previously (22). All of the cell culture media and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). LNCaP and CV-1 cells were obtained from American Type Culture Collection (Manassas, VA), and the PSA ELISA assay kit was purchased from ICN Pharmaceuticals (Orangeburg, NY).

Cell Culture and Transfection. Human prostate cancer LNCaP cells were maintained in RPMI medium supplemented with 8% fetal bovine serum, essential amino acids, and sodium pyruvate. Monkey Kidney CV-1 cells were grown in MEM plus 8% fetal bovine serum, essential amino acids, and sodium pyruvate. Cells were seeded on 25-cm^2 tissue culture flasks a day before transfection. Lipofectin-mediated transfection was performed essentially as described (22). Transfection was stopped after 5 h by replacing the DNA/lipofectin mixture with fresh medium containing charcoal-stripped serum and incubated overnight, allowing cells to recover. Cells were then trypsinized and seeded on 96-well plates the next morning. After cells were attached, hormones were added, and the cells were incubated for 16 h before assaying. Luciferase and β-galactosidase activities were determined as described (22).

Cell Proliferation Assays. LNCaP cells were seeded in 24-well plates with $\sim 4 \times 10^4$ cells/well and maintained in phenol red-free RPMI plus 8% charcoal-stripped serum for 3 days. On day 3, cells were treated with fresh medium containing different concentrations of compounds, and the medium was replaced every other day for 6 days. Cell numbers were determined at the end of 6 days by DPA DNA assays (23, 24).

DPA DNA Assays. The DPA DNA assay was performed as described with minor modification (23, 24). Medium was removed by suction from attached LNCaP cells. Prechilled 20% hyperchloric acid and 0.16% acetaldehyde were mixed at a 5:1 ratio and added to each well at 120 μl/well. Subsequently, 200 μl of 4% DPA/acetic acid were added. The plates were sealed with parafilm and incubated at room temperature overnight. Color development was measured with a microtiterplate reader with absorption wavelength 595 nm and reference wavelength 750 nm.

PSA ELISA. LNCaP cells were seeded at a density of 1×10^5 cells/well in phenol red-free RPMI plus 8% charcoal-stripped serum and incubated for 3 days. Fresh medium (500 μl) containing the hormones to be tested were added on day 3 and incubated with the cells for 20 h. For the PSA ELISA, 50 μl of medium from each well were used, and total PSA secreted in the medium was determined using a PSA enzyme immunoassay test kit (ICN Pharmaceuticals) following the manufacturer's protocol. Total PSA secreted in the medium was normalized to total DNA content (measured by DPA DNA assay) in the wells.

RT-PCR. Monolayer LNCaP cells were maintained in phenol red-free RPMI plus 8% charcoal-stripped serum for 3 days and then induced with different concentrations of DHT, DOC, or Dex for 24 h. Total RNA was collected using Ultraspec RNA isolation system (Biotecx, Houston, TX), and RT-PCR was carried out with Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA). Primer sets used in RT-PCR were: β-actin-5′: 5′-TGTGATGGTGGGAATGGGTCAG-3′, β-actin-3′: 5′-TTTGATGTCACGCACGATTTC-3′, PSA-617: 5′-CCTCCTGAAGAATCGATTCC-3′, and PSA-814: 5′-GAGGTCCACACACTGAAGTT-3′ (25).

RESULTS

Earlier studies have identified progesterone, estradiol, and certain weak adrenal androgens as potent activators of the AR T877A mutant. In this study, we tested a broader spectrum of compounds for their ability to activate either the wild-type or the T877A mutant AR in transient transfection assays in CV-1 cells using the MMTV-Luc reporter gene. Compounds tested include several adrenal androgens, their precursors and metabolites, and various clinically relevant glucocorticoids. In a pilot screen, we found that many compounds, at a concentration of 1 μM, could activate the reporter gene in cells transfected with the mutant AR (Fig. 1*A*) while exhibiting little or no activity in cells transfected with the wild-type AR or with a control plasmid (data not shown). It was surprising to us that the mutant AR could be activated by such a structurally diverse group of ligands. This suggested that resistance to antihormonal treatment may reflect a gain-of-function property of the AR mutant, wherein ligands which would not normally activate AR can do so on the mutant. Of particular importance was the observation that the mutant AR could be activated by a series of naturally occurring and synthetic glucocorticoids. Because both AR and the GR can activate the MMTV-Luc reporter gene, it was important to confirm that the reporter gene was activated by the transfected AR and not by the low level of endogenous GR present in this cell system. This was addressed by assaying the ability of the selected compounds to induce an activating conformational change in the structure of AR. In a previous study, we reported the identification of several short peptides which interact with AR when it is in a transcriptionally active conformation (22).[4] One of these peptides, D30, contains a leucine-rich LxxLL motif that is often present in coactivators, facilitating their interaction with the receptor ligand-binding domain (26). To evaluate the interaction between the D30 peptide and AR, we made use of a mammalian two-hybrid system. The D30 peptide was fused to the DNA-binding domain of the yeast protein Gal4 (Gal4-DBD), and the AR and its mutant were modified by insertion of a viral acidic activation domain VP16 at their NH_2 terminus. When VP16-AR is recruited to the DNA-bound Gal4DBD-D30 in the cells, it reconstitutes the transcriptional activity of the Gal4-VP16 and drives the expression of a cotransfected reporter gene containing five copies of the Gal4-response elements. Using this assay, we found that the ability of compounds to activate MMTV-Luc, through either AR or AR/T877A, correlates with their ability to effect an activating conformational change within these proteins. With this assay, we confirmed that compounds unexpectedly found to activate MMTV-Luc in our assay system were acting through AR (Fig. 1*B*).

The potentially important finding that compounds other than androgens could function as activating ligands for the mutant AR prompted us to perform dose-response curves to determine whether the concentrations required for AR activation are within the known physiological/pharmacological range of these compounds. For these studies, we limited analysis to those compounds which were likely to have the most clinical relevance in prostate cancer patients (Table 1). The results of this transcriptional assay indicate that the EC_{50} for

[4] Chang, C-Y. and McDonnell, D. P., unpublished data.

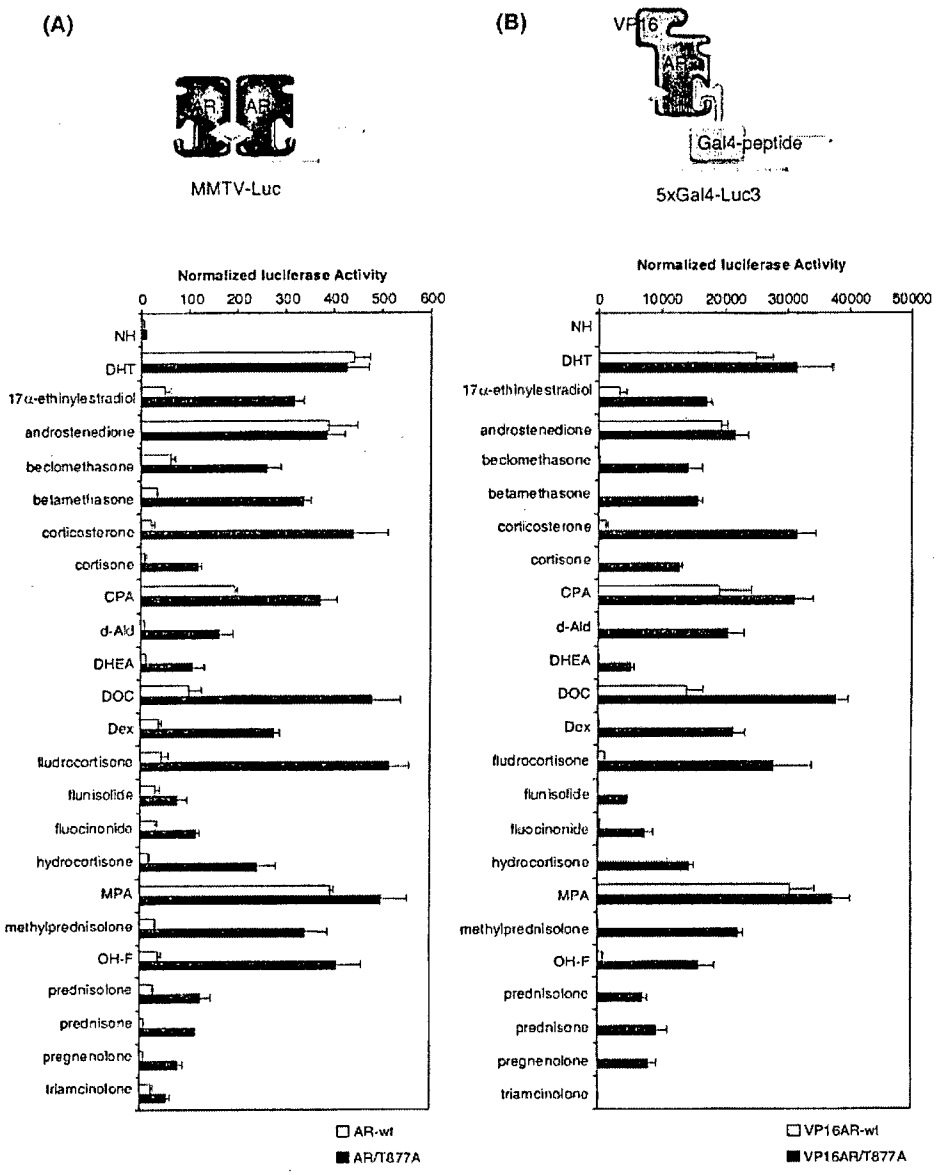

Fig. 1. A large number of structurally diverse compounds can activate the AR/T877A mutant. In *A*, CV-1 cells were transfected with either the wild-type AR or the AR/T877A mutant expression plasmids and the reporter gene MMTV-Luc together with a normalization vector pCMV-βgal. Transfected cells were induced with 1 μM each compound for 16 h, then the luciferase and β-galactosidase activities were measured. In *B*, CV-1 cells were transfected with the pM-D30 plasmid expressing the D30 peptide fused to the Gal4DBD and either VP16AR-wild type or VP16AR/T877A together with the reporter gene *5xGal4Luc3* and pCMV-βgal. Transfected cells were treated and assayed as in *A*. *NH*, no hormone; *CPA*, cyproterone acetate; *d-Ald*, d-aldosterone; *DHEA*, dehydroepiandrosterone; *MPA*, medroxyprogesterone. Normalized luciferase activity was obtained by dividing the luciferase activity by the β-galactosidase activity. The values shown are the mean ±SD of three determinations. The results shown are representative of multiple experiments performed under the same conditions.

DOC and Dex are 2.8×10^{-10} and 6.4×10^{-8} M, respectively, both being within achievable serum concentrations (Fig. 2). A number of other compounds, including corticosterone and fludrocortisone, are also potent agonists of the mutant AR, exhibiting EC_{50} values of 7.7×10^{-9} and 2.6×10^{-8} M, respectively. Corticosterone and DOC are produced mainly in the zona glomerulosa of the adrenal cortex as intermediary products in the biosynthesis of aldosterone (27). The production of DOC is up-regulated in a number of disease states and is not affected by orchiectomy or by the treatment with GnRH agonists or antagonists (28–30). Dex is often used in patients with advanced disease who have failed standard hormonal therapies.

The compounds which showed potential clinical relevance were further analyzed for their ability to up-regulate PSA production in prostate cancer cells. PSA, an AR-regulated, secreted glycoprotein produced mainly in the prostate, is a validated marker used to track the progression of prostate cancer (31). We chose LNCaP cells, a cell line derived from the bone metastasis of a patient with advanced prostate cancer, which carries the T877A mutation, for these studies. Semiquantitative RT-PCR of mRNA prepared from LNCaP cells treated with DOC and Dex indicated that these two compounds can induce the accumulation of PSA mRNA at concentrations similar to what we determined to be optimal in the reporter gene assays in CV-1 cells (Fig. 3). Furthermore, using an ELISA assay, it was observed that cells treated with these compounds also secreted increased amounts of

Table 1 *EC_{50} of different compounds on activation of wild-type and T877A mutant AR*[a]

Compounds	AR/wild type	AR/T877A
DHT	2.1×10^{-11} M	1.2×10^{-11} M
OHF	$>10^{-7}$ M	1.0×10^{-9} M
Corticosterone	$>10^{-7}$ M	7.7×10^{-9} M
DOC	$>10^{-7}$ M	2.8×10^{-10} M
Aldosterone	$>10^{-7}$ M	$>10^{-7}$ M
Fludrocortisone	$>10^{-7}$ M	2.6×10^{-8} M
Hydrocortisone (cortisol)	$>10^{-7}$ M	$>10^{-7}$ M
Cortisone	$>10^{-7}$ M	$>10^{-7}$ M
Beclomethasone	$>10^{-7}$ M	$>10^{-7}$ M
Betamethasone	$>10^{-7}$ M	$>10^{-7}$ M
Dexamethasone	$>10^{-7}$ M	6.4×10^{-8} M

[a] CV-1 cells were transfected with either wild-type or T877A mutant AR expression plasmid together with MMTV-Luc and pCMV-βgal. Different concentrations of hormones (10^{-13}–10^{-7} M) were added 16 h before assaying. The data were plotted, and EC_{50} was calculated using the software Prism 3 (Graphpad, San Diego, CA).

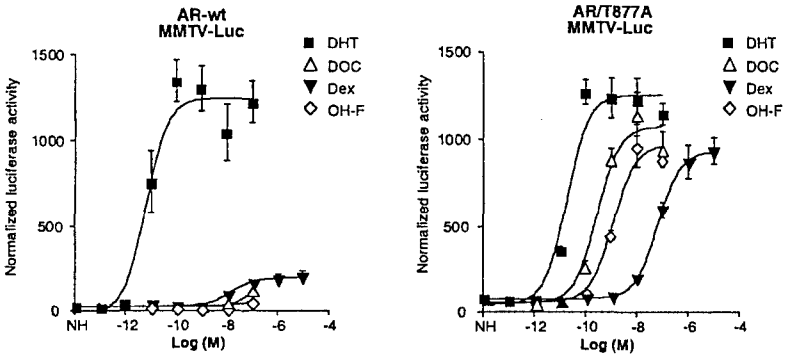

Fig. 2. CV-1 cells were transfected with expression plasmids for either the wild-type AR or AR/T877A mutant and the reporter gene *MMTV-Luc,* along with a normalization vector pCMV-βgal. Transfected cells were induced with different concentrations of compounds for 16 h, and the luciferase and β-galactosidase activities were measured. Normalized luciferase activity was obtained by dividing the luciferase activity by the β-galactosidase activity. The values shown are the mean ± SD of three determinations. The results shown are representative of multiple experiments performed under the same conditions.

PSA into the medium when compared with untreated cells (Fig. 4*A*). Although it has been shown that the LNCaP cells do not express estrogen receptor, progesterone receptor, and GR (32, 33), we included the antiandrogen bicalutamide in the assay to ensure that the response observed was indeed mediated by the AR but not other receptors. We found that cotreatment with 10 μM bicalutamide was able to significantly suppress the PSA secretion induced by all of the compounds tested, confirming the involvement of AR in this pathway (Fig. 4*B*). The same concentration of bicalutamide has no inhibitory effect on Dex-induced GR transcriptional activity (Fig. 4*C*).

Androgens are mitogenic in LNCaP cells (33, 34). We decided, therefore, to determine whether DOC and Dex, two compounds that are potent activators of the AR/T877A mutant, can induce LNCaP cell proliferation. We used a modified DPA assay to measure the total DNA content as an index of cell number. In 6-day proliferation assays, DHT and OH-F induced a ~2.5-fold increase in cell number, in agreement with previously published reports (Refs. 32 and 33; Fig. 5*A*). DOC at concentrations of <1 nM can induce significant cell proliferation with maximum induction being reached at ~10 nM. Interestingly, the biphasic dose-response curve seen with DHT and other steroids (32, 33) was not observed when cells were treated with DOC and OH-F. Our studies, thus far, have not produced an explanation for this phenomenon. Dex, although a potent AR agonist in AR/T877A-dependent gene transcription and in induction of PSA secretion, failed to stimulate significant cell proliferation under the conditions of our assay. When we treated the cells with increasing concentrations of Dex in the presence of 0.1 nM DHT, we found that high concentrations of Dex can actually inhibit DHT-induced cell proliferation (Fig. 5*B*). However, Dex did not inhibit cell proliferation down to the same level as the no hormone-treated control but rather to a level similar to what Dex alone would achieve at that concentration.

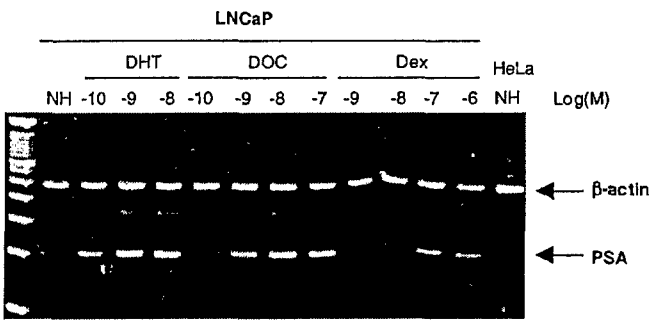

Fig. 3. Both DOC and Dex induce PSA mRNA expression in LNCaP cells. LNCaP cells were maintained in RPMI media containing charcoal-stripped serum for 3 days. Fresh media containing different concentrations of DHT, DOC, and Dex were added to the cells on day 4 and incubated for 24 h. Total RNA was isolated from cells and used in RT-PCR. Primer sets specific for PSA and β-actin produced 214- and 515-bp PCR products, respectively. Total RNA from HeLa cells was used as a negative control.

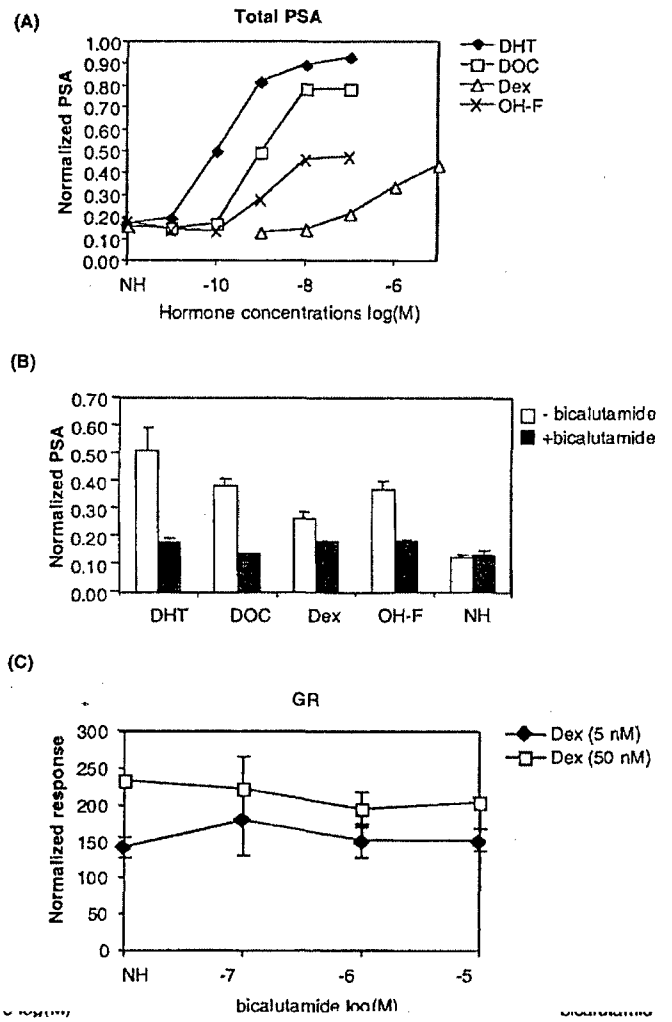

Fig. 4. Both DOC and Dex induced PSA secretion in LNCaP cells. LNCaP cells were maintained in RPMI media containing charcoal-stripped serum for 3 days. In *A*, fresh media containing different concentrations of DHT, DOC, and Dex were added to the cells on day 4 and incubated for 24 h. Media (50 μl) was removed from each treatment group and analyzed using a PSA-ELISA assay kit. In *B*, media containing 1 nM DHT, 10 nM DOC, 5 μM Dex, or 50 nM OH-F in the presence or absence of 10 μM bicalutamide were added to the cells and incubated for 24 h. Secreted PSA was measured as in *A*. Normalized PSA value was determined by dividing the total PSA secreted in the medium by the total DNA content in the well. The values shown are the mean ±SD of three determinations. In *C*, CV-1 cells were transfected with GR expression plasmid together with MMTV-Luc and pCMV-βgal. After transfection, cells were treated with various concentrations of bicalutamide in the presence of either 5 or 50 nM Dex. Luciferase and βgal activities were analyzed 16 h after hormone addition.

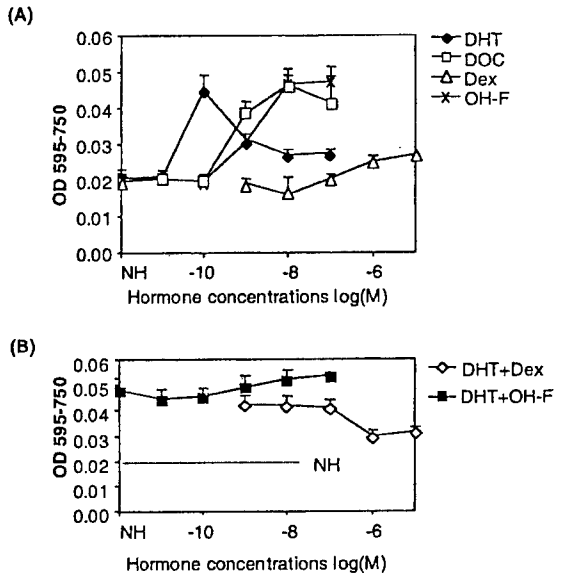

Fig. 5. DOC induces LNCaP cell proliferation. LNCaP cells were maintained in RPMI media containing charcoal-stripped serum for 3 days. In *A*, cells were treated with media containing different concentrations of DHT, DOC, Dex, and OH-F on day 4, and the media were changed every other day for 6 days. In *B*, cells were treated with media containing 0.1 nM DHT in the presence of different concentrations of Dex or OH-F on day 4, and the media were changed every other day for 6 days. Cell numbers at the end of the 6-day treatment were determined using a DPA assay. The values shown are the mean ±SD of three determinations. The results shown are representative of multiple experiments performed under the same conditions.

This indicated to us that with respect to proliferation, Dex functions more like a weak partial agonist.

DISCUSSION

In this study, we have identified a panel of nonandrogenic activators of the AR/T877A mutant, an AR mutant frequently found to occur in late stage prostate cancers. These compounds can activate AR-mediated gene transcription at concentrations that are achievable either in normal physiological states or after administration as a drug. DOC is an endogenous hormone that is normally present in relatively low levels in the circulation, though high enough to activate AR/T877A. DOC is also elevated in certain disease states and after administration of some pharmaceuticals. Of note in this regard is ketoconazole, a drug which was used initially to treat hormone-refractory prostate cancer based on its ability to inhibit C17,20-desmolase and steroid 17α-hydroxylase, enzymes involved in the biosynthesis of adrenal androgens (35, 36). In addition, ketoconazole inhibits 11-β hydroxylase (37), leading to increased plasma levels of DOC (14-fold) and corticosterone (3-fold) in prostate cancer patients treated with high-dose ketoconazole (38). Therefore, ketoconazole or other mechanistically related pharmaceuticals may have the potential for adverse effects in patients whose tumor characteristics are consistent with those having an AR/T877A mutation. Interestingly, when aminoglutethimide, an agent which inhibits adrenal steroidogenesis, is administered to patients with documented flutamide withdrawal, a response is seen in 48% of patients, whereas <5% respond in the absence of the withdrawal phenomenon (39). This suggests that, in patients who have this mutant receptor, adrenal glucocorticoid intermediates might exert a stimulatory effect.

Dex is used therapeutically in many prostate cancer contexts; it can relieve bone pain, diminish ureteric obstruction, and provide temporary decompression of metastasis to the epidural space compressing the spinal cord in advanced prostate cancer patients. It is also frequently prescribed as a pituitary suppressant to reduce the production of adrenal androgens in patients who have failed hormonal therapy and as an antiemetic in patients undergoing chemotherapy and/or radiation therapy. Our results show that Dex activates the AR/T877A-mediated gene transcription at a dose that is achievable in the clinical setting; however, a much higher concentration of Dex is required to induce PSA secretion and cell proliferation in LNCaP cells. We believe that this may be attributable to the fact that higher levels of AR are produced in the transient transfection system, which allows the dose-response curve to be shifted to the left. Indeed, in the LNCaP cells, Dex does not activate the endogenous AR/T877A mutant-mediated reporter gene expression as efficiently as in the CV-1 system (data not shown). However, the responsiveness to Dex can be restored by increasing expression of an exogenous AR/T877A in LNCaP cells (data not shown). As noted earlier, amplification of AR has been observed in a substantial number of specimens (30%) from patients who have failed AA (9–11). These observations, coupled with our data which shows that the Dex-bound AR/T877A can acquire a transcriptionally active conformation, suggest that Dex may be able to function as an androgen in some prostate cancer cells. Therefore, we speculate that some tumors that have become refractory to prior flutamide treatment may have a less favorable response to subsequent Dex treatment.

Zhao *et al.* (19) have also recently described the loss of androgen-dependence in tumors containing the L701H mutation, which allows the AR to be activated by cortisol and cortisone. Combining our data, one might speculate that in so-called androgen-independent prostate cancer, glucocorticoids may stimulate tumor cell growth by an AR-mediated mechanism.

Our data demonstrate that the T877A mutation allows the AR to be activated by multiple endogenous hormones and pharmaceuticals, therefore circumventing the need for androgens to support tumor cell growth. Certain clinical strategies for treatment of advanced prostate cancer bear reassessment based on this work: (*a*) the use of TAA therapy; (*b*) the use of high-dose ketoconazole as second-line hormonal therapy; and (*c*) the indiscriminate use of Dex in a variety of contexts. The results of this study highlight the complexity of AR pharmacology and reinforce the need to use mechanism-based approaches in the search for new hormonal therapies for the treatment of prostate cancer.

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Ligand-Selective Interactions of ER Detected in Living Cells by Fluorescence Resonance Energy Transfer

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Some aspects of ligand-regulated transcription activation by the estrogen receptor (ER) are associated with the estrogen-dependent formation of a hydrophobic cleft on the receptor surface. At least *in vitro*, this cleft is required for direct interaction of ER with an α helix, containing variants of the sequence LXXLL, found in many coactivators. In cells, it is unknown whether ER interactions with the different LXXLL-containing helices are uniformly similar or whether they vary with LXXLL sequence or activating ligand. Using fluorescence resonance energy transfer (FRET), we confirm in the physiological environment a direct interaction between the estradiol (E2)-bound ER and LXXLL peptides expressed in living cells as fusions with spectral variants of the green fluorescent protein. This interaction was blocked by a single amino acid mutation in the hydrophobic cleft. No FRET was detected when cells were incubated with the antiestrogenic ligands tamoxifen and ICI 182,780. E2, diethylstilbestrol, ethyl indenestrol A, and 6,4′-dihydroxyflavone all promoted FRET and activated ER-dependent transcription. Measurement of the level of FRET of ER with different LXXLL-containing peptides suggested that the orientations or affinities of the LXXLL interactions with the hydrophobic cleft were globally similar but slightly different for some activating ligands. (*Molecular Endocrinology* 16: 487–496, 2002)

THE ERα AND ERβ ARE members of a large class of nuclear receptors that regulate the transcription of genes in response to binding small molecule ligands (1–3). The regulatory roles of ER in disorders like breast cancer and osteoporosis make it an important therapeutic target (4–9). One of the signature features of the ER-targeting compounds is that they may have different stimulatory or repressive effects depending on the cellular context. For instance, the breast cancer drug tamoxifen is an antiestrogen in breast tissue but, in the uterus, it mimics the estrogenic activity of the physiological hormone, E2 (7, 10). Other compounds, such as the osteoporosis drug raloxifene, show a different clinical profile (11). Improved designer estrogens with higher selectivity for specific tissues would permit tissue-specific, estrogen-regulated disorders to be treated with minimal side effects (12, 13).

The mechanisms by which E2 and the selective ER modulators (SERMs) show tissue-specific activities remain unresolved but are at least partially related to ligand-regulated alterations in ER structure and function. In both its unliganded and liganded state, the ER is part of larger complexes with other accessory proteins (14). These accessory proteins, some of which are still unidentified, can stabilize ER structure and regulate transcription at different DNA effector sites (15). Ligand binding causes a conformation change in the ER (16, 17), which alters the affinities of the receptor for these accessory proteins (18, 19). One possible mechanism for SERM activity is that different ligands recruit different sets of accessory proteins and thereby differentially regulate gene transcription (20–22). Differential cofactor interactions, together with tissue-dependent expression of ERα, ERβ, and each cofactor, could explain tissue-selective SERM activity.

To elucidate, and ultimately predict, differential SERM action, it is therefore essential to measure the ligand-induced, direct interactions between the ER and different accessory proteins in the cellular environment (20–22). Many coactivators that interact with the E2-activated ER contain one or more copies of a consensus sequence, LXXLL (L, leucine; X, any amino acid) (23, 24). Structural studies have shown that an

Abbreviations: CFP, Cyan fluorescent protein; DES, diethylstilbestrol; DHF, 6,4′-dihydroxyflavone; EIA, indenestrol A; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; L, leucine; RFP, red fluorescent protein; SERM, selective ER modulator; X, any amino acid.

isolated LXXLL peptide will interact with a hydrophobic cleft that forms on one surface of the E2-bound ER (16). This hydrophobic cleft constitutes the activation function AF-2, which is conserved amongst nuclear receptors and participates in ligand-regulated gene transcription (25). Two-hybrid interaction assays have proved very useful for identifying and characterizing the ligand-regulated interactions of LXXLL-containing factors and peptides with ER expressed in cells (20–22). However, two-hybrid assays measure only whether proteins interact, and not whether they interact with differing structural characteristics or affinities.

We applied a microscope-based assay using fluorescence energy resonance transfer (FRET) to measure in living cells the ability of a ligand to modulate LXXLL interactions with ER. FRET measures the proximity of two molecules as a consequence of the degree to which the fluorescence energy excited in a donor fluorophore, linked to one factor, is not emitted and instead is nonradiatively transferred to an acceptor fluorophore, linked to another factor (26–30). We observed in the cellular environment that ERα, fused to the red fluorescent protein (RFP) interacted directly with LXXLL peptides, fused to the cyan (CFP) or green (GFP) fluorescent proteins. These interactions were promoted by E2 but blocked by tamoxifen and another SERM, ICI 182,780, which confirmed prior studies in two-hybrid (21) and fluorescence colocalization (31) assays. Like E2, the synthetic ligands diethylstilbestrol (DES), ethyl indenestrol A (EIA), and 6,4′-dihydroxyflavone (DHF) promoted FRET between ERα-RFP and two different LXXLL peptides fused to GFP. All these interactions were dependent upon the integrity of AF-2 within the ligand binding domain of ERα. E2, DES, EIA, and DHF yielded similar levels of FRET for the interaction of ER with one LXXLL peptide. However, small, ligand-selective differences in the level of FRET were measured for interaction with the other LXXLL target sequence. This indicated that there were subtle, ligand-specific, and LXXLL-specific differences in the orientation or affinity of LXXLL interaction with ER. The accurate measurement of such nuances in the interactions of ER in the cellular environment will help distinguish the similarities and cell-type dependent differences in ligand-selective ER activities.

RESULTS

Fluorescent Protein-Tagged ERα and LXXLL for FRET Measurements

Isolated LXXLL sequences retain the ability to interact specifically with estrogen-bound ER (16, 21). In our initial studies, the 19-amino acid-long LXXLL-containing sequence F6 (21), previously shown to form a complex with ERα (21, 31), was fused to the carboxy terminus of CFP. X-ray crystallographic structures of LXXLL bound to ER (16) predict that, if LXXLL binds directly to ER, the CFP fluorophore should project toward RFP fused to the carboxy terminus of ERα. This positioning would be optimal for FRET from the CFP donor to the RFP acceptor.

The ERα-RFP fusion was transcriptionally active (Fig. 1). ERα-RFP or control expression vectors were transfected into ER-deficient HepG2 cells together with either of two different, E2-sensitive promoters controlling the expression of a luciferase reporter. One promoter consisted of three copies of an estrogen response element linked to a minimal TATA box (32). This reporter defines the "classical" activities of ERα-RFP, in which estrogen response is mediated by direct ER interaction with a single DNA binding site in the promoter. The second promoter, from the complement 3 gene (C3), contains three suboptimal ER binding elements, which together allow ER to bind and regulate transcription in response to E2 (33).

Two days after transfection, promoter activity was assessed by measuring the amount of luciferase expressed in extracts of cells grown in E2-deficient media or in parallel cells treated with 10^{-6} M E2. Both the 3xERE (Fig. 1, *black bars*) and C3 (Fig. 1, *white bars*) promoters were activated upon E2 addition. In contrast, tamoxifen and ICI 182,780 did not activate ERα-RFP at either promoter, even though wild-type ERα weakly activated the C3 promoter in the presence of tamoxifen, but not ICI 182,780 (22, 33). Thus, ERα-RFP was defective in tamoxifen activation. Because the estrogenic activities of E2 were not disrupted by the fusion of RFP to the carboxy terminus of ERα, ERα-RFP remained viable for studying agonist activation via AF-2.

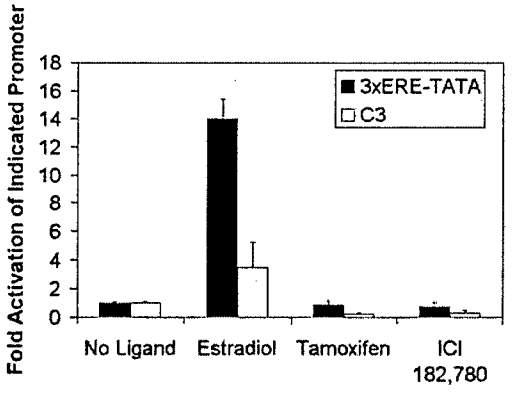

Fig. 1. E2-Regulated Activation of Two Promoters by the ERα-RFP Fusion Protein

HepG2 cells, grown in E2-free media, were transfected with the ERα-RFP expression vector and either of two reporter plasmids expressing luciferase under the control of E2-responsive promoters. The 3xERE-TATA and complement C3 promoters were activated upon incubation of the cells with E2, but not by the SERMs tamoxifen or ICI 182,780.

Controls for the Accurate Measurement of FRET between CFP-LXXLL and ERα-RFP

The measurement of FRET relies on the accurate quantification of the amount of fluorescence emitted by the donor and acceptor fluorophores upon donor excitation (29). The donor CFP is excited optimally by blue light to emit light of energy in the blue-green (cyan) wavelength, whereas the RFP acceptor emits red light upon excitation by light of mid-visible wavelengths, including cyan. If the cyan fluorescent CFP is in close proximity to RFP (<100 Å apart), some of the fluorescence energy from CFP will be absorbed by, and excite, RFP. Thus, when excited by blue light, energy transfer from CFP to RFP would decrease the emission of cyan light and increase the emission of red light.

For controls, we first quantified the amount of fluorescence in cells that independently expressed ERα-RFP or CFP-LXXLL. Expression vectors encoding ERα-RFP or CFP-LXXLL were transfected into GHFT1–5 pituitary progenitor cells grown in estrogen-free media and plated onto microscope coverslips. GHFT1–5 cells contain endogenous ERα, but promoter responses to E2 in GHFT1–5 cells are not significantly altered upon ERα overexpression (34, 35). Because overexpression of ERα in GHFT1–5 cells does not reduce ER response as it does in many other cell types (36), the actions of expressed ER measured in GHFT1–5 cells likely mimic those of endogenous receptors. GHFT1–5 cells also have a flat morphology, which facilitates data collection by fluorescence microscopy (31, 37). The transfected cells were treated with E2, or other ligands as discussed later, or with the control vehicle (ethanol). After allowing 24 h for expression, the amounts of fluorescence emitted from the control cells separately expressing ERα-RFP and CFP-LXXLL were measured in the cyan, red, and FRET channels by quantitative fluorescence microscopy. Digital images from cells expressing CFP-LXXLL were collected by specifically exciting CFP with light of wavelengths between 431 and 434 nm and collecting emissions between 455 and 480 nm (Fig. 2A, *cyan*). Digital images from the ERα-RFP control cells were collected by 550–560 nm excitation and 580–630 nm emission (Fig. 2B, *red*).

The excitation/emission parameters for CFP and RFP resulted in little bleedthrough fluorescence, respectively, in the control cells expressing ERα-RFP and CFP-LXXLL (Fig. 2B, *cyan*; Fig. 2A, *red*). This bleedthrough was accurately quantified by marking each nucleus containing CFP-LXXLL or ERα-RFP as a contiguous assembly of pixels containing more fluorescence than the background. The total amount of cyan and red fluorescence above the background fluorescence was then measured within the nucleus of each control cell. Red fluorescence from 27 different E2-treated cells expressing only CFP-LXXLL was negligible: on average, 0.0009 ± 0.0026 the amount of cyan fluorescence. This means that the amount of

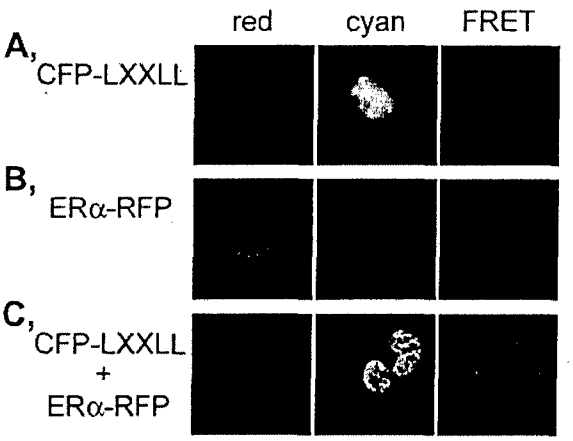

Fig. 2. FRET Microscopy of LXXLL Interactions with ERα

GHFT1–5 cells grown in estrogen-free media were transfected with the A, CFP-LXXLL expression vector; B, ERα-RFP expression vector or C, both vectors together, then incubated with E2 (shown) or other ligands (not shown). Digital fluorescent images were collected using *red*-, *cyan*-, or FRET-selective excitation and emission filters. Coexpression of CFP-LXXLL with ERα-RFP causes the LXXLL peptide to occupy the intranuclear location of ERα in estrogen-treated cells (31).

energy emitted by CFP in the red channel was statistically insignificant. Similarly, the bleedthrough of ERα-RFP into the cyan channel was 0.0016 ± 0.0019 the amount of emission in the red' channel. These ratios were similar regardless of the ligand treatment for each cell (not shown). Although the amount of CFP-LXXLL or ERα-RFP expressed in each transiently transfected cell varied, plotting the amount of bleedthrough as a function of the amount of CFP-LXXLL or ERα-RFP fluorescence in each cell (Fig. 3, A and B, *open boxes*) showed that these ratios were consistently measured regardless of the amount of CFP-LXXLL or ERα-RFP expressed.

Ligand-Regulated FRET between CFP-LXXLL and ERα-RFP

As described previously (31), expression of ERα caused the coexpressed LXXLL peptide to colocalize with the E2-bound ERα, whereas the LXXLL peptide, by itself, distributed throughout the cell (Fig. 2). To determine if there was a hormone-regulated, direct interaction of LXXLL with colocalized ERα, we measured FRET between coexpressed CFP-LXXLL and ERα-RFP. The low level of CFP and RFP bleedthrough enabled us to selectively and accurately measure the amounts of CFP-LXXLL and ERα-RFP coexpressed in the same cell. These values are then used to correct for the contributions of the known amounts of CFP and RFP to the FRET channel, as discussed below.

In cells coexpressing CFP-LXXLL and ERα-RFP, FRET was detected, upon blue light excitation, as an increase in acceptor fluorescence transferred from the

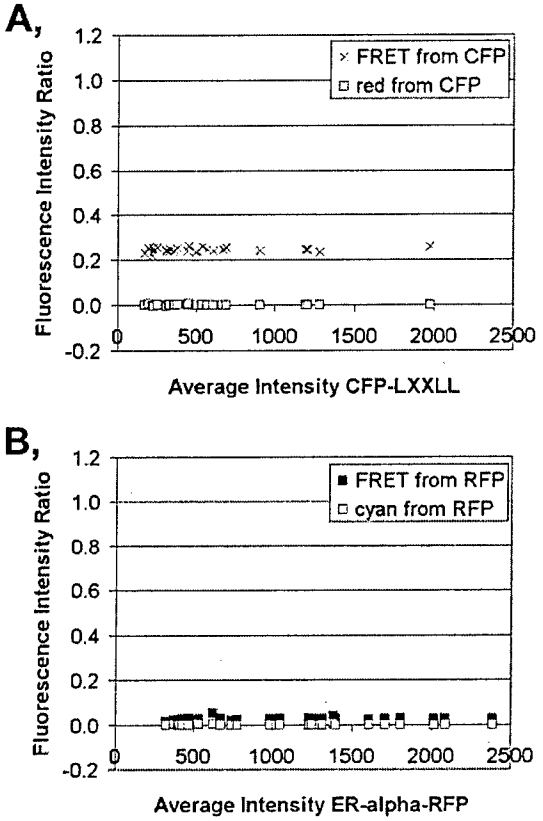

Fig. 3. Contribution of CFP-LXXLL and ERα-RFP to Each Excitation/Emission Channel

Total background-subtracted fluorescence from each nucleus was quantified in each channel for each digital image. A, The amount of fluorescence measured in the red channel when only CFP-LXXLL was expressed (red from CFP) was minimal (0.09%, on average, of the amount of fluorescence in the cyan channel). 24.55% of the cyan fluorescent of CFP-LXXLL alone bled through into the FRET channel (FRET from CFP). B, Fluorescence bleedthrough of ERα-RFP-expressing cells in the cyan (cyan from RFP) and FRET (FRET from RFP) channels was 0.16% and 2.88%, respectively. These values were constant regardless of the amount of CFP-LXXLL or ERα-RFP transiently expressed in these cells. This demonstrates the accuracy by which these physical constants for each fluorophore was measured.

donor (FRET channel: 431–434 nm excitation/580–630 emission) relative to a decrease in donor fluorescence (cyan channel: 431–434/455–480). Therefore, FRET was measured as an increase in the ratio of FRET/cyan fluorescence from a cell expressing both CFP-LXXLL and ERα-RFP relative to the FRET/cyan ratios emitted from independently expressed CFP-LXXLL and ERα-RFP. In the control CFP-LXXLL-expressing cells, the amount of bleedthrough fluorescence into the FRET channel was 0.2455 ± 0.0094 that emitted in cyan channel (Fig. 2A), which did not vary with the overall amount of CFP-LXXLL in the cell (Fig. 3A, Xs). The bleedthrough of ERα-RFP fluorescence to the FRET channel was 0.0288 ± 0.0066 that emitted in the red channel (Fig. 2B), which also did not vary with the amount of ERα-RFP present in each cell (Fig. 3B, *black boxes*).

To calculate the relevant FRET/cyan from donor ratio, we first calculated the amounts of CFP-LXXLL and ERα-RFP present in the coexpressing cells. This was achieved by subtracting the minor contributions of CFP to the red channel (0.09% the value of cyan fluorescence using the matched control data in the prior section) and of RFP to the cyan channel (0.16% the value of the corrected red channel). We then subtracted the contribution of RFP to the FRET channel (2.88% the value of the corrected red channel). This remaining signal in the FRET channel contained the CFP bleedthrough to the FRET channel plus any sensitized emissions that resulted from the transfer of energy from CFP to RFP. If there was no FRET, the FRET/donor (remaining FRET/corrected cyan, hereafter FRET/cyan) ratio remained that of the donor CFP alone (0.2455). However, if there was transfer of energy from CFP to RFP, the amount of CFP fluorescence decreased and the amount of FRET increased, so that the FRET/cyan ratio increased.

The FRET/cyan ratio averaged from 32 E2-treated cells coexpressing CFP-LXXLL and ERα-RFP increased to 0.5412 ± 0.2018. Because cells with low amounts of CFP-LXXLL relative to ERα-RFP have fewer CFP donors in close proximity to the RFP acceptor, the FRET/cyan ratio varied with the relative amounts of RFP and CFP fluorescence measured in each cell. To account for this variation, the FRET/cyan ratio was graphed against the relative amounts of bleedthrough-corrected cyan and red fluorescence for each E2-treated cell (Fig. 4, *black boxes*). The slope of this graph was linear and consistent between experiments, indicating that the acceptor (RFP)-driven level of FRET within each cell was a constant. If the LXXLL peptide were not attached to CFP (Fig. 4, *gray triangles*) or if CFP-LXXLL and ERα-RFP coexpressing cells were treated with the antiestrogen ICI 182,780 instead of E2 (Fig. 4, *white boxes*), the FRET/cyan ratio remained identical to the 0.2455 FRET/cyan ratio of CFP-LXXLL alone (Fig. 4, Xs at acceptor/donor = 0) regardless of the relative amounts of CFP-LXXLL and ERα-RFP measured in the cell. This validated our calculations and demonstrated the accuracy with which we measure the energy transfer. Thus, we observed a ligand-regulated direct interaction of an LXXLL peptide with ERα in living cells.

Ligand-Regulated, AF-2-Dependent FRET between GFP-LXXLL and ERα-RFP

To further validate our FRET measurements, we repeated the FRET studies of ERα-RFP with the same F6 LXXLL peptide, but labeled with GFP instead of CFP. Control measurements similar to those described above for the CFP-LXXLL construct were conducted to determine the bleedthrough of GFP-LXXLL fluorescence into the red and FRET channels. These GFP-LXXLL bleedthrough constants, and bleedthrough constants

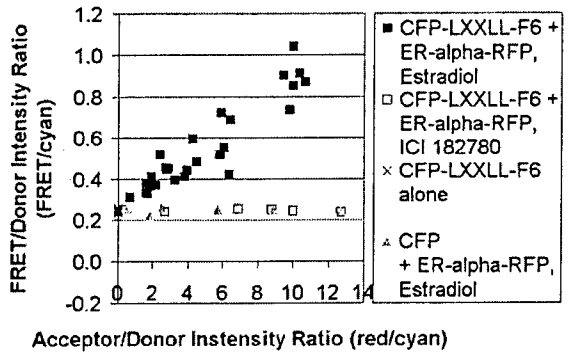

Fig. 4. Estrogen-Specific Interaction of CFP-LXXLL and ERα-RFP in Living Cells

Background-subtracted fluorescence from each nucleus was corrected for the bleedthrough values of red from CFP, cyan from RFP, and FRET from RFP (see *Results*). The remaining amount of fluorescence in the FRET channel isolates the contribution from the donor CFP to the FRET channel. It also contains emissions resulting from any energy transferred from CFP to RFP. Productive FRET also is accompanied by a decreased emission in the donor, cyan, channel. Thus, the FRET/cyan ratios from the isolated donor were calculated for each nucleus using the bleedthrough-subtracted values to determine the amount of energy transferred. These FRET/cyan ratios were plotted against the amount of acceptor (red) relative to donor (cyan). When the cells are treated with E2 (*black boxes*), the FRET/cyan ratio increased linearly with the red/cyan ratio at a slope characteristic of the efficiency of energy transfer from CFP to RFP. In contrast, the FRET/cyan ratio was not different from CFP-LXXLL alone (Xs) if the cells were treated with the SERM ICI 182,780 (*white boxes*) or if the LXXLL peptide was removed from CFP (*gray triangles*).

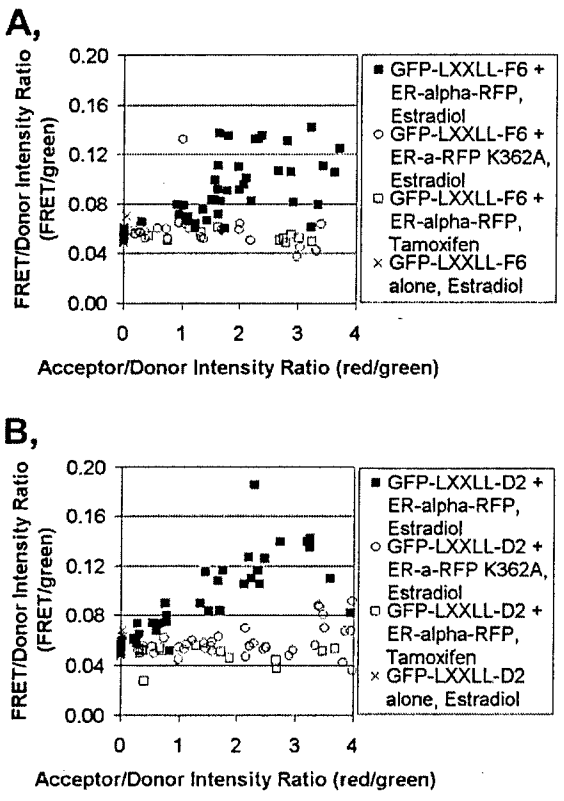

Fig. 5. AF-2-Dependent Interaction of Two Different LXXLL Peptides with ERα in Living Cells

FRET/Donor ratios were calculated for ERα-RFP interactions with two different LXXLL peptides (21, 31) attached to GFP: A, F6 (same as in Fig. 4) and B, D2. The FRET/donor ratio increased with the acceptor/donor ratio in cells treated with E2 (*black boxes*), but not tamoxifen (*white boxes*). Mutation of lysine 362 in the hydrophobic AF-2 cleft of ERα resulted in a loss of FRET (*white circles*) indicating that AF-2 was required for the direct interaction of LXXLL with ERα-RFP measured by FRET in living cells. Similar measurements were made for both peptides for different SERMs (Table 1).

determined from parallel ERα-RFP control cells, were used to calculate the amount of ligand-regulated FRET in cells coexpressing GFP–LXXLL and ERα-RFP. Coexpressing cells treated with tamoxifen showed a FRET/donor (FRET/green) ratio of 0.0530 ± 0.0043 (n = 18) (Fig. 5A, *white boxes*), that was not significantly different from the 0.0541 ± 0.0039 ratio measured in the control cells expressing GFP-LXXLL alone (Fig. 5A, Xs).

After treatment with E2, cells containing both ERα-RFP and GFP-LXXLL showed a FRET to green ratio of 0.0971 ± 0.0291 (n = 44) that varied proportionally to the RFP/GFP ratio (Fig. 5A, *black boxes*). This confirmed that E2 promotes an interaction between ERα and LXXLL in living cells, whereas tamoxifen does not. When E2-treated cells coexpressing ERα-RFP and GFP not containing the 19-amino acid LXXLL peptide were analyzed, the FRET/GFP ratio remained at 0.0549 ± 0.0098 (n = 14). In addition, mutation of a single lysine in the hydrophobic cleft of ERα to alanine (K362A) abolished FRET of GFP-LXXLL with ERα-RFP in E2-treated cells (Fig. 5A, *white circles*) as the FRET/GFP ratio remained as 0.0557 ± 0.0063 (n = 27). This demonstrated that the cleft, which is essential for E2-dependent transcription via AF-2 (25), is required for direct interaction of LXXLL with ERα in the physiological environment of the living cell.

In the presence of E2, the FRET/green ratio from coexpressed wild-type ERα and LXXLL increased with the RFP/GFP ratio in the cells (Fig. 5A), confirming that *bona fide* FRET was detected. The slopes of these graphs, summarized in Table 1 as the range of slopes encompassing the 95% confidence intervals, emphasized that interaction of the F6 LXXLL peptide with ERα was promoted by E2 (slope = 0.017 to 0.021), but not by the SERMs tamoxifen (slope = −0.001 to 0.001) or ICI 182,780 (slope = −0.002 to 0.000). Thus, FRET precisely measured a ligand-specific, AF-2-dependent direct interaction between ERα and an LXXLL peptide in living cells.

Ligand-Specific Interactions of Different LXXLL Peptides Binding to AF-2 in ERα

FRET measurements are highly sensitive to distance between the fluorophores, and fall off to the sixth

Table 1. Ninety-Five Percent Confidence Intervals in Slopes and Y-Intercepts of FRET/Donor *vs.* Donor/Acceptor Graphs (RFP/GFP <4)

	GFP-LXXLL-F6		GFP-LXXLL-D2	
	Slope	Y-intercept	Slope	Y-intercept
No ERα-RFP	No slope	0.053–0.055	No slope	0.053–0.055
ERα-RFP wt				
No hormone	0.007–0.011	0.053–0.057	0.007–0.009	0.053–0.056
E2	*0.017–0.021*	0.053–0.058	*0.022–0.026*	0.053–0.058
Tamoxifen	−0.001–0.001	0.054–0.055	−0.005–0.003	0.053–0.055
ICI 182,780	−0.002–0.000	0.054–0.055	−0.001–0.002	0.054–0.055
DES	*0.016–0.021*	0.052–0.057	*0.018–0.023*	0.053–0.057
EIA	*0.017–0.021*	0.053–0.056	*0.014–0.018*	0.053–0.057
DHF	*0.017–0.021*	0.053–0.056	**0.029–0.034**	0.052–0.055
ERα-RFP K362A				
No hormone	−0.004–0.001	0.054–0.057	−0.005–0.003	0.054–0.055
E2	−0.003–0.001	0.054–0.059	0.001–0.003	0.053–0.056
DES	−0.004–0.002	0.054–0.056	0.002–0.006	0.053–0.056
EIA	−0.001–0.002	0.053–0.056	0.000–0.003	0.054–0.056
DHF	−0.007–0.002	0.054–0.056	0.000–0.003	0.054–0.055

power as the distance between them increases (28, 29). The distance dependency of FRET would, in principal, allow the detection of small conformational differences between interacting molecules. We therefore measured the level of FRET between ERα-RFP and another 19-amino acid-long, LXXLL-containing peptide, "D2" (21), fused to GFP. D2 contains sequences flanking the LXXLL motif that differ from those in the F6 peptide.

Control measurements established that the FRET/GFP and RFP/GFP ratios for GFP-LXXLL-D2 alone were no different from those measured for GFP-LXXLL-F6 (not shown). When coexpressed with ERα-RFP, GFP-LXXLL-D2, like GFP-LXXLL-F6, showed E2- and AF-2-dependent FRET that was not promoted by tamoxifen (Fig. 5B). In the presence of saturating (10^{-6} M) E2, the slopes of the FRET/GFP *vs.* RFP/GFP graphs (reported as 95% confidence intervals) were similar, but slightly different, for GFP-LXXLL-F6 (Table 1, 0.017–0.021) and GFP-LXXLL-D2 (0.022–0.026). This suggested that the D2 and F6 peptides bound to the hydrophobic cleft of E2-bound ERα with marginal differences in orientation or with slightly different affinities.

Using FRET to Distinguish SERM-Regulated Interactions

The ability of FRET to measure subtle differences in the direct interactions of the LXXLL motif and ERα in living cells could be used as a sensitive new assay for detecting specific activities of new SERMs *in vivo*. All ligands that trigger LXXLL motif binding to ERα are known to strongly activate transcription at promoters containing the classical ERE promoter element. Therefore, a compound that elicits a strong level of FRET between ERα-RFP and GFP-LXXLL in this assay might also activate transcription of an ERE-driven gene in a reporter assay.

To test this hypothesis, we synthesized and tested two synthetic ligands of uncharacterized estrogenic or antiestrogenic activities that were reported previously to bind ER with high affinity (38–40): ethyl indenestrol A and 6,4′-dihydroxyflavone (Fig. 6). These compounds and a known ER agonist diethylstilbestrol were compared with E2 for their abilities to elicit FRET in cells coexpressing ERα-RFP and GFP-LXXLL-F6 or GFP-LXXLL-D2. E2, DES, EIA, and DHF all were able to trigger significant levels of FRET between ERα-RFP and both LXXLL peptides (Table 1, slopes, *italic*). All of these ligand-regulated interactions were blocked upon mutation of lysine 362 in ERα to alanine (Table 1, ERα-RFP K362A), indicating that LXXLL was interacting directly with the hydrophobic pocket of ERα in each case.

For interaction of ERα-RFP with GFP-LXXLL-F6, the levels of FRET activated by E2, DES, EIA, and DHF were not statistically different ($P > 0.05$) (Table 1, GFP-LXXLL-F6 slopes). Similarly, the levels of FRET determined for GFP-LXXLL-D2 interaction with ERα-RFP in the presence of DES and EIA were not significantly different than those observed with GFP-LXXLL-F6 (Table 1). In contrast, DHF activated a significantly greater level of FRET with the D2 LXXLL peptide than with the F6 LXXLL peptide (Table 1, *boldface*). Thus, all compounds promoted the direct interactions of two different LXXLL peptides with ERα in the cellular environment, but precise FRET measurements allowed subtle variations in those interactions to be observed.

The similar levels of FRET with the F6 peptide suggested that the ER-binding compounds EIA and DHF both caused ERα to adopt a conformation that permitted LXXLL-F6 to bind into the hydrophobic AF-2 cleft in the same orientation as occurs when E2 or DES binds to ERα. Because reporter gene assays show that DES and E2 activate transcription from a classical ERE in an AF-2-dependent fashion, the similar AF-2/LXXLL-F6 interactions adopted by the EIA and DHF-bound ERs suggested that these compounds might activate transcription at an ERE site. Reporter gene

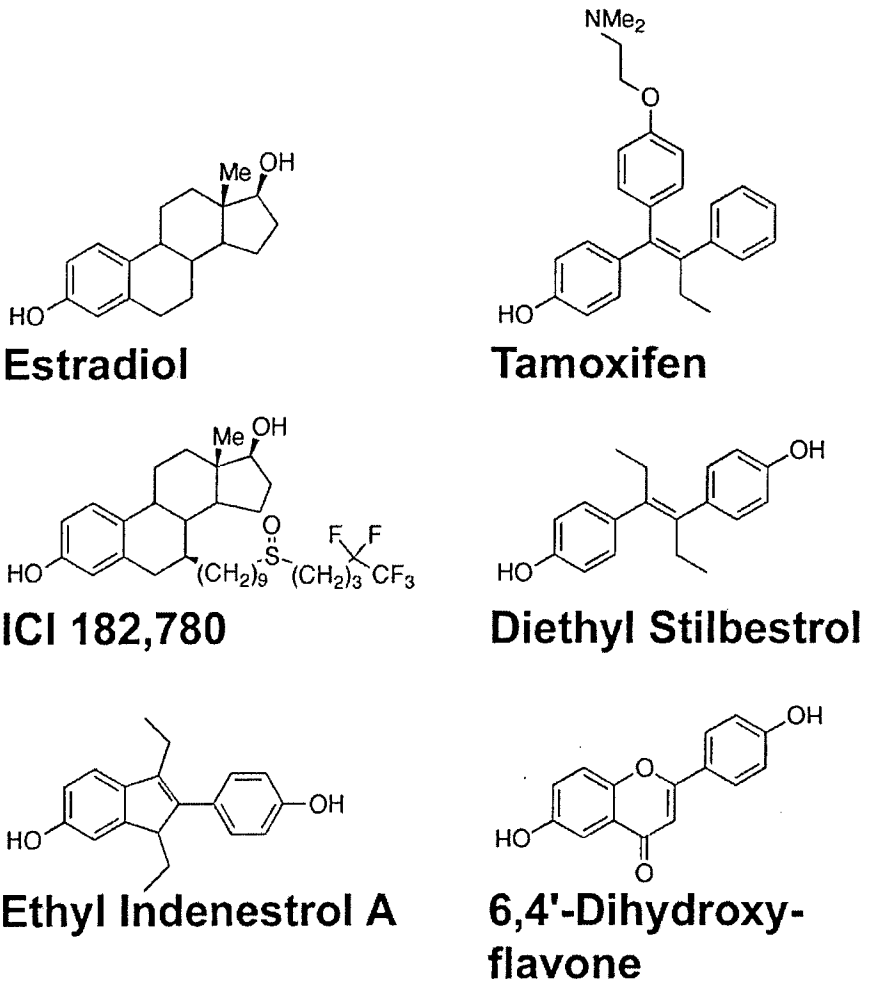

Fig. 6. Structures of the ER-Binding Compounds Used in this Study

The phenolic ring common to all compounds is oriented to the *left*. There are two different phenolic rings in ethyl indenestrol A and 6,4′-dihydroxyflavone, for which only one of the orientations is shown.

assays were performed in HeLa cells using transiently transfected wild-type ERα and a luciferase gene driven by the classical ERE from the vitellogenin promoter (Fig. 7). The promoter was activated upon expression of unliganded ERα. This activation was blocked by the SERM raloxifene, which acts as an antiestrogen for AF-2-dependent transcription (41). Incubation with 10^{-5} M EIA and 10^{-5} M DHF both activated transcription from an ERE site as effectively as 10^{-5} M E2. Thus, the ability of two different LXXLL peptides to productively interact with ERα AF-2 in living cells was associated with agonist activity of four separate ligands, each with a distinct chemical structure.

DISCUSSION

The ligand-regulated interactions of a receptor with its cofactors are fundamental to nuclear receptor action (3, 15, 42–44). These interactions are commonly detected with *in vitro* column-binding assays that rely on the interactions of purified proteins in artificial buffers. Alternatively, two-hybrid assays detect an ill-defined cellular association between two proteins as the activation of a downstream reporter gene. As more is understood about the mechanisms underlying nuclear receptor activation, new challenges are arising to effectively and efficiently measure those interactions, particularly in living cells (31, 45–49).

Only recently has FRET been used to measure protein-protein interactions in the environment of living eukaryotic cells (28, 49, 50). FRET has been used to detect nuclear receptor interactions with cofactor fragments, labeled with spectral variants of GFP (49). We show here that LXXLL peptides by themselves are sufficient to interact, in an agonist-regulated fashion, directly with ERα in the cellular environment (Table 1). Moreover, these interactions are wholly dependent upon AF-2 in ERα. A weak interaction of LXXLL with ERα was also detected in the absence of ligand. This

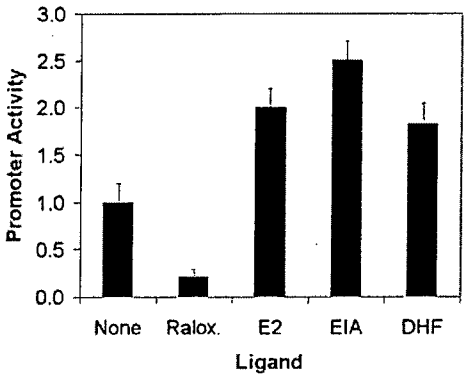

Fig. 7. ER Binding Compounds that Promote FRET also Activate a Simple ERE-TATA Promoter

HeLa cells transfected with an expression vector for wild-type ERα and with an E2-regulated promoter were treated with different ligands. Raloxifene blocked promoter activation by expressed ER interacting with estrogens in the cell culture media. E2, EIA, and DHF all caused further activation of the promoter.

ligand-independent interaction was blocked by the same K362A mutation in AF-2 that abrogated agonist-dependent FRET. Thus, in living cells, both the ligand-independent and agonist-dependent interactions of LXXLL with ERα are dependent upon AF-2.

Our approach allowed us to precisely quantify the level of FRET between the interacting factors. Because the amount of FRET falls very rapidly, to the sixth power, with the separation of the fluorophores (28–30), differences in the relative spatial orientations of ER and LXXLL affect the amount of FRET measured in each complex. In our studies, the efficiency of FRET between ERα and a given LXXLL peptide was similar for each ligand. However, there were subtle differences, particularly with 6,4′-dihydroxyflavone for which the level of FRET was higher for ERα interaction with LXXLL-D2 than with LXXLL-F6. The slight variations in the levels of FRET indicate subtly different ligand-specific interactions. Different levels of FRET may suggest that the LXXLL peptide is bound to AF-2 in a different position such that the GFP and RFP fluorophores are different distances apart. Alternatively, the on- or off-rates for the interaction of LXXLL with ERα may be different, leading to quantitatively different levels of FRET. For instance, the higher level of LXXLL FRET with all liganded ERs than with the unliganded ER is consistent with prior observations that agonist binding dramatically stabilizes the LXXLL interaction with ERα (31, 51).

The FRET studies described here, by themselves, showed the similarities and differences in LXXLL interactions with ERα bound by four different activating ligands and two different AF-2-blocking ligands. Other techniques may complement the FRET studies of ligand-selective nuclear receptor action. Determining the kinetics of fluorescence recovery after photobleaching fluorophore-linked nuclear receptors (46, 48) and cofactors (48) at isolated regions within the nucleus may help to determine if the ligand-selective changes in FRET efficiency arise from altered kinetics in the LXXLL interaction with ERα. Measuring the recovery of FRET after selectively photobleaching the interacting cofactor would more precisely correlate fluorescence recovery with direct interactions with ERα in the cellular environment.

The benefits of drugs that regulate nuclear receptor activities in some tissues are often counteracted by unwanted receptor actions in other tissues. It is therefore important to identify compounds with desirable selective modulatory properties (12, 13, 20–22, 41, 52). However, most current assays for interaction are insufficient to distinguish the tissue-selective actions of new compounds from previously existing SERMs (22, 41). The precise measurement of ER/cofactor interactions afforded by FRET will allow the detection, in different cellular environments, of more subtle differences in the interactions of ER, or any other nuclear receptor, bound to different ligands. This will aid the development of clinically effective compounds that regulate specific interactions in specific cell types. Indeed, we found that FRET between ER and two LXXLL-containing peptides was useful in predicting the E2-mimicking activity of two previously untested ER-binding compounds, EIA and DHF. However, different levels of FRET for the LXXLL interactions with AF-2 suggest that these compounds possess somewhat distinct properties. This precise quantification of FRET between nuclear receptors and conformation-specific peptide probes developed by us (20–22) and others (53) will greatly contribute to a better mechanistic understanding of estrogen action and may be potentially useful for discovering SERMs with improved tissue-selective actions.

MATERIALS AND METHODS

Analysis of Estrogen-Regulated Promoter Activation

The 3xERE-TATA-Luc and C3-Luc estrogen-responsive promoters have been previously described (32, 33). The carboxy terminus of human ERα was fused, in frame with an eight-amino acid linker, to the amino terminus of RFP by inserting a PCR-generated ERα cDNA into the *Nhe*I and *Bam*HI sites of pDsRed1-N1, an RFP expression vector (CLONTECH Laboratories, Inc., Palo Alto, CA). The ERα-RFP expression vector was cotransfected with either the 3xERE-TATA-Luc or C3-Luc reporters into HepG2 cells and the transfected cells were treated with E2, tamoxifen, ICI 182,780 or ethanol control vehicle as previously described (21). Cells were then lysed and the amount of luciferase activity in the extracts was measured as previously described (21).

The synthesis, ER binding properties and transcriptional activation profiles of EIA and DHF will be reported elsewhere. Transfection conditions and assay protocols used for the testing of EIA and DHF with wild-type human ERα and the ERE reporter gene assay in HeLa cells (Fig. 7) were identical to those reported previously (41, 54).

Cellular Imaging

GHFT1–5 cells were transfected with the ERα-RFP expression vector and doxycycline-inducible GFP-LXXLL or CFP-LXXLL expression vectors as previously described (31). Transfected cells were grown for 24 h in estrogen-free media containing 3–5 μg/ml doxycycline. A total of 10^{-6} M of each ligand was then added and the cells grown for a further 24 h before data collection. Quantitative fluorescence images were collected with a Hamamatsu ORCA cooled interline camera attached to an Olympus Corp. IX-70 microscope controlled by Universal Imaging Corp. (Downingtown, PA) Metamorph software. Filter combinations, described in the Results section, were obtained from Chroma Technology Corp. (Brattelboro, VT). Fluorescence quantification of marked nuclei and background was performed using Metamorph software. Background subtractions and bleedthrough corrections were applied using Microsoft Excel (Microsoft Corp., Redmond, WA).

FRET Analysis

FRET measurements can be accomplished using relatively affordable fluorescence microscopic equipment and image collection software. Control measurements of the cells separately expressing the donor or the acceptor fluorophore (Fig. 2) indicate the point at which the user's equipment no longer accurately quantifies the fluorescence ratios critical for FRET determination. Only cells containing donor and acceptor fluorescence amounts greater than those that are accurately measured are included for calculating FRET. Because the ratios measured are physical parameters of the fluorophores, FRET measurements are highly consistent between separate experiments provided that all parameters affecting the relative ratios of fluorescence quantification in the donor, acceptor and FRET channels are kept constant. This includes using the same 1) objective lens, 2) relative integration times for the different channels, 3) dichroic mirror, 4) excitation/emission filters, and 5) camera.

Average fluorescence ratios ± SD were calculated from data collected on multiple days using Microsoft Excel. Ninety-five percent confidence intervals in the slopes and Y-intercepts of the FRET/donor *vs.* acceptor/donor graphs were calculated using GraphPad Software, Inc. Prism software (San Diego, CA). Only data up to an acceptor/donor ratio of 4 were included in the calculation of the slopes for GFP-LXXLL FRET with ERα-RFP. The linearity of the graphs tended to decrease beyond this point, as the amount of acceptor became more saturating. This acceptor/donor ratio of 4 should not be used by others as a defined parameter as it depends on the nature of the molecular interaction (our unpublished data) and the ability of the user's equipment used to quantify the specific acceptor and donor fluorescence.

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Evaluation of Ligand-Dependent Changes in AR Structure Using Peptide Probes

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Mutations in the AR are frequently found in relapsed prostate cancers, some of which permit antiandrogens as well as nonandrogenic compounds to function as androgens. However, the molecular mechanism(s) by which these mutations enable this aberrant AR pharmacology is still unknown. To explore this issue, we used a series of LxxLL-containing peptides (L, leucine; x, any amino acid) to probe the conformation of the AF-2/coactivator binding pocket of AR and AR mutants when complexed with different ligands. We have identified in a previous study two peptides that bind to the wild-type AR in an agonist-dependent manner. Interestingly, we found these same peptides also interacted with several AR variants that are frequently found in antihormone refractory prostate cancers, in the presence of either androgens or antiandrogens. This suggests that the agonist activity of antiandrogens and other physiologically relevant ligands occurs because they, in the background of these mutations, allow AR-AF2 to adopt an active conformation. Initially, this result appeared to contradict the findings of others that suggest that coactivator binding to AR-AF2 is not required for AR activity. In probing this paradox further, however, we determined that the role of AR-AF2 appears to be to stabilize the overall structure of the receptor, allowing the amino terminus to interact with appropriate coactivators. This conclusion is supported by our finding that overexpression of the AF2-binding peptides blocks the interaction between the amino and carboxyl termini of AR but does not attenuate AR transcriptional activity. This can be explained by the fact that overexpression of the LxxLL-containing peptide or the amino terminus of AR appears to have a similar effect on the AR-ligand binding domain, as both have the ability to stabilize agonist binding by decreasing ligand off-rate. Thus, we believe that resistance in certain prostate cancers occurs as a consequence of receptor mutations that enable antagonist-and/or nonclassical ligand-bound AR to present a wild-type-like AF-2 conformation. (*Molecular Endocrinology* 16: 647–660, 2002)

THE AR belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors that modulate diverse biological functions in response to either endogenous or exogenous stimuli (1, 2). Members of this gene family share sequence homologies and exhibit similar modular domain structures (3–5). Nuclear receptors contain a carboxyl-terminal ligand-binding domain (LBD) that allows them to bind their cognate ligands, and a central DNA binding region (DBD) that permits their interaction with specific DNA sequences located within the promoter regions of the target genes that they regulate. In most receptors, the LBD also harbors a transactivation function domain (AF-2), a key protein-protein interaction module that allows these receptors to direct the assembly of multiprotein transcription complexes at target genes. Those receptors that bind with high affinity to steroid hormones such as androgens, estrogens, glucocorticoids, mineralcorticoids, and progestins, also contain at least one additional activation function domain (AF-1) at the amino terminus of the receptor. The two AF domains in these receptors can either synergize or function independently to enable the regulation of gene transcription in a cell-type and/or tissue-specific manner (6–8).

In the past few years, it has become clear that the nuclear receptors do not function alone and that the activities they manifest are the result of their ability to interact with other cellular coregulator proteins, coactivators, and corepressors (9–11). Structural and biochemical studies have determined that upon binding their cognate hormone, the AF-2 of the nuclear receptors undergoes an activating conformational change, enabling the receptor to interact with various coactivators (12, 13). Most of the validated coactivators contain a leucine-rich LxxLL-motif (L, leucine; and x, any amino acid) that mediates their interactions with the receptor AF-2 (14). Crystallographic studies using the ERα as a model revealed that when ER is activated by an agonist, the LxxLL-motif from the coactivator GRIP-1 (glucocorticoid receptor interacting protein-1) binds in a hydrophobic surface atop the ligand-binding pocket (12). It appears that most receptors have evolved to utilize this hydrophobic surface as a major docking site for coactivators, and not surprisingly mu-

Abbreviations: AF-1, Activation function-1; DBD, DNA binding region; DHT, 5α-dihydrotestosterone; GRIP-1, glucocorticoid receptor interacting protein-1; L, leucine; LBD, ligand binding domain; OHF, hydroxyflutamide; SRC-1, steroid receptor coactivator-1; x, any amino acid.

tations of residues that line this hydrophobic pocket generally render the receptor transcriptionally inactive. Similarly, changing the conserved leucine residues in the LxxLL-motif into alanine in the known coactivators abrogates their ability to interact and potentiate receptor activity. Indeed, the LBD (containing the AF-2) of most receptors when tethered to DNA is able to induce reporter gene transcription in an agonist-dependent manner, for it in itself is capable of recruiting the LxxLL-containing coactivators. With that being said, some of these coactivators have been shown to interact with multiple sites on the receptor. SRC-1 (steroid receptor coactivator-1) and GRIP-1, for example, can interact with both the AF-1 and AF-2 of PR and ER using different regions of the protein (15–17). The physiological significance of the alternate contact points between receptor and coactivators remains to be determined.

In contrast to other nuclear receptors, the AR-LBD when expressed alone manifests minimal transcriptional activity. Although the full-length AR has been shown to interact also with SRC-1 and GRIP-1, unlike their interaction with other nuclear receptors, the LxxLL-motif in these coactivators appears not to be sufficient for these interactions (8, 18, 19). In addition, it has also been reported that the C terminus of the p160s can interact and potentiate the AR AF-1 (20, 21). It is believed therefore that AR may have unique coactivator binding characteristics. Various AR-interacting proteins have been identified by yeast two-hybrid screens using different AR fragments as bait, including ARA70, ARA54, ARA55, ARA24, ARA160, FHL-2, ARIP3, ARIP4, etc. (22–28). These proteins bind to different regions of AR and when overexpressed in cells potentiate AR activity. The physiological significance and the detailed mechanisms of action of these proteins, however, remains to be determined.

Protein-protein interactions govern almost all biological processes, including activation and repression by nuclear receptors. Not surprisingly, analysis of the ligand-induced structural alterations in the receptor, and how they influence its ability to interact with comodulators, has become one of the most important areas in the study of receptor pharmacology. Crystallographic studies of the ER-LBD suggest that the conformational changes which occur upon agonist binding permit the docking of the coactivators through their LxxLL motifs (12, 29, 30). Upon antagonist binding, on the other hand, receptor conformation is altered so that the helix-12 of the LBD is repositioned to occupy the coactivator-binding pocket, thereby blocking the access of coactivators. These studies provide a structural basis for the agonist and antagonist activities of receptor ligands. The crystal structure of the agonist-bound AR-LBD is very similar to that of the other nuclear receptors (31). Still unpublished is the structure of antagonist-bound AR; therefore, the conformational differences between agonist- and antagonist-activated receptor, and the impact of these structural alterations on receptor function, are not known. In addition, many antiandrogen-resistant prostate tumors contain one or more mutations in the AR-LBD, which allows the receptor to manifest an aberrant ligand-specificity. For instance, mutation of the codon 877 from threonine to alanine permits this mutant AR to activate gene transcription in the presence of hydroxyflutamide, an antagonist of the wild-type receptor (32, 33). The crystal structure of this mutant AR-LBD revealed that the replacement of threonine 877 with alanine enlarges the ligand binding pocket enabling the accommodation of the bulky side chains on the C17 of steroids (34). This may explain why this mutant receptor can bind to a variety of other hormones that normally do not interact with AR (31, 34, 35). Clearly, conformational changes within the AR-LBD have a significant impact on receptor pharmacology. Without a structure of the antagonist-bound receptor, however, it is still unclear how a compound can switch from being an antagonist to an agonist, as has been observed on the AR/T877A mutant.

In a previous study, we used a series of short peptides to probe ligand-induced conformational changes in ER (36–38). This peptide binding analysis complemented ongoing crystallographic studies and revealed that different ER ligands allow the presentation of different protein-protein interaction surfaces on ER and facilitate the interaction of the receptor with different coregulatory proteins. Most importantly, this work also led to the discovery of at least one mechanism to explain how antiestrogen-resistant tumors may develop in breast cancers, where the tumor cells switch from recognizing antiestrogens as antagonists to agonists. In this current study, we present data using a similar approach to the study of mechanisms underlying agonist/antagonist activities of AR-ligands, a first step in the development of new antiandrogens for the treatment of prostate cancer and other endocrinopathies.

RESULTS

Probing AR Conformation Using LxxLL-Containing Peptides

In a previous study, we used phage display to assess ligand-induced structural changes within the ER and identified several peptide probes whose binding to the receptor is conformation sensitive (36, 38, 39). Some of these peptides contain an LxxLL-motif and recognize agonist-activated ER but not that activated by antagonists. Because these peptides were found to be capable of interacting with several other nuclear receptors, we wished to determine if any of them could be used to recognize the active conformation of the AR. We found only two peptides, D11 and D30, that were capable of interacting with AR in the presence of the agonist 5α-dihydrotestosterone (DHT). In the current study, we characterized these peptides further

and assessed the interaction of AR with each peptide in the presence of either the agonist DHT, or two different antagonists: hydroxyflutamide (OHF) and bicalutamide. A mammalian two-hybrid assay was performed to evaluate the interactions between AR and all of the LxxLL-containing peptides identified previously (38, 40). For this assay we expressed each peptide as a Gal4-DBD fusion and used a modified AR, VP16-AR, in which the acidic VP16 activation domain was cloned onto the extreme amino terminus of AR. Interactions between AR and the peptides were detected by measuring the expression of a luciferase reporter gene containing five copies of the Gal4-response element. The results of a representative analysis are shown in Fig. 1. While these ERα- and/or ERβ-interacting peptides were shown to interact with multiple other nuclear receptors (38, 40), we were able to identify only two peptides (D11 and D30) that could interact with agonist-activated AR (Table 1). Interestingly, AR did not interact with the LxxLL-motifs contained within SRC-1 and GRIP-1 in this assay, in agreement with previous findings by other investigators (8, 18, 19). This indicated to us that the protein-protein interaction surfaces presented on AR are unusual and that the receptor may regulate transcription using a distinct set of coactivators. Nevertheless, the D11 and D30 peptides specifically interacted with agonist- but not antagonist-activated AR, suggesting that these two peptides do indeed detect an active conformation of this receptor. Although the D11 and D30 peptides both contain an "SSRLxxLLM" motif, we do not know as yet which amino acids are required for AR-binding. Such an analysis has been confounded by the recent identification of another LxxLL peptide in our laboratory (LX23), which interacted

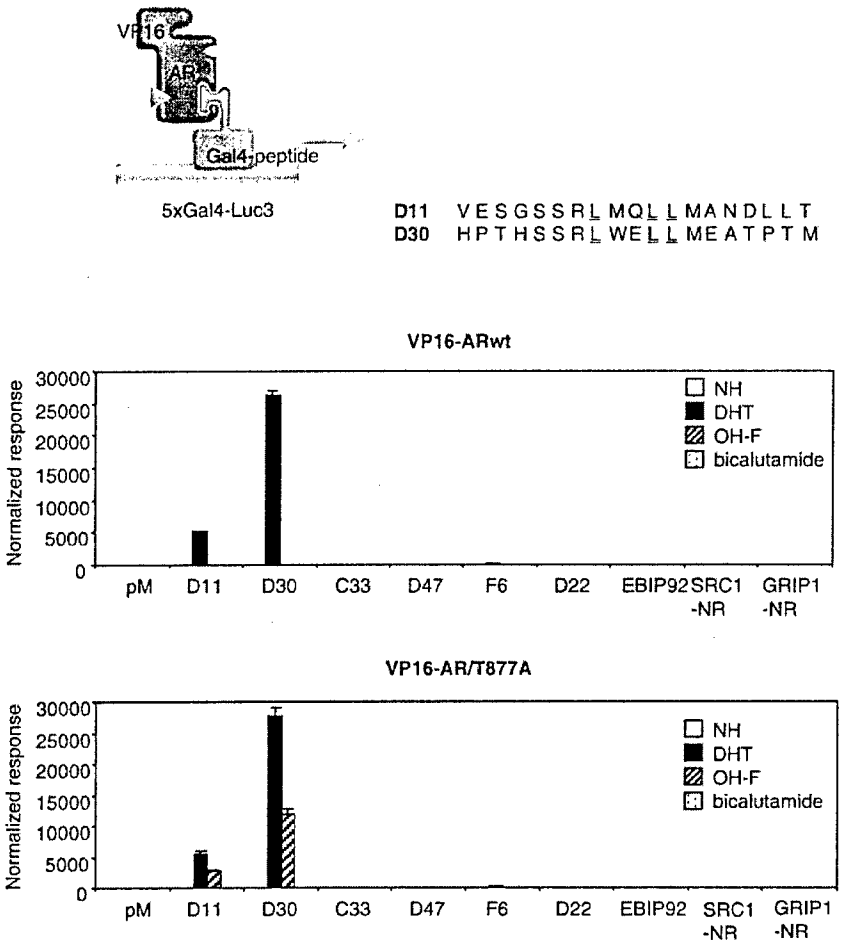

Fig. 1. The T877A Mutation Permits Antiandrogen-Bound AR to Adopt a Conformation Similar to that of the Wild-Type Receptor

Different LxxLL-motif containing peptides were fused to the Gal4-DBD, and the full-length ARs (wild-type and the T877A mutant) were modified to include a VP16-activation domain at their amino termini. Interactions between peptides and AR were determined by measuring the expression of a reporter gene containing five copies of the Gal4-response elements. CV-1 cells were transfected with different peptide-Gal4DBD constructs together with either the VP16-ARwt or the VP16-AR/T877A expression plasmid, and reporter constructs 5xGal4Luc3 and pCMV-βgal. After transfection, cells were treated with either vehicle control (NH), 100 nM DHT, 100 nM OHF, or 1 μM bicalutamide for 16 h. Luciferase activity was measured and normalized to the activity of the coexpressed β-galactosidase.

Table 1. The AR Interacts with Only a Small Subset of LxxLL-Containing Peptides

Peptide	Sequence	AR Interaction
D11	VESGSSRLMQLLMANDLLT	+
D30	HPTHSSRLWELLMEATPTM	+
ER4	SSNHQSSRLIELLSR	−
D14	QEAHGPLLWNLLSRSDTDW	−
D47	HVYQHPLLLSLLSSEHESG	−
C33	HVEMHPLLMGLLMESQWGA	−
F6	GHEPLTLLERLLMDDKQAV	−
D22	LPYEGSLLLKLLRAPVEEV	−
D48	SQWENSILYSLLSDRVSLD	−
D43	AHGESSLLAWLLSGEYSSA	−
D40	SGWNESILYRLLQADAFDV	−
D15	PSGGSSVLEYLLTHDTSIL	−
F4	PVGEPGLLWRLLSAPVERE	−
RIP140/935-944	VLKQLLLSEN	−
C5	TVWERASLADLLEWQEEVR	−
293	SSIKDFPNLISLLSR	−
EBIP37	TGGGVSLLLHLLNTEQGES	−
EBIP41	RRDDFPLLISLLKDGALSQ	−
EBIP44	YGLKMSLLESLLREDISTV	−
EBIP45	MSYDMLSLYPLLTNSLLEV	−
EBIP51	FPAEFPLLTYLLERQGMDE	−
EBIP96	VESEFPYLLSLLGEVSPQP	−
EBIP49	VSSEGRLLIDLLVDGQQSE	−
EBIP53	DTPQSPLLWGLLSSDRVEG	−
EBIP60	GGTQDGYLWSLLTGMPEVS	−
EBIP66	SLPEEGFLMKLLTLEGDAE	−
EBIP70	VMGNNPILVSLLEEPSEEP	−
EBIP76	VLVEHPILGGLLSTRVDSS	−
EBIP87	QTPLLEQLLTEHIQQG	−
EBIP92	SVWPGPELLKLLSGTSVAE	−
EBIP56	GSWQDSLLLQLLNRTELMA	−
GRIP-1 NR1[a]	DSKGQTKLLQLLTTKSDQM	−
GRIP-1 NR2[a]	LKEKHKILHQLLQDSSSPV	−
GRIP-1 NR3[a]	KKKENALLRYLLDKDDTKD	−
SRC-1 NR1[a]	YSQTSHKLVKLLTTTAEQQ	−
SRC-1 NR2[a]	LTARHKILHRLLQEGSPSD	−
SRC-1 NR3[a]	ESKDHQLLRYLLDKDEKDL	−
FxxLF[b]	SKTYRGAFQNLFQSVREVIQNP	+

[a] The NR boxes from SRC-1 and GRIP-1 were not tested individually but as Gal4DBD fusion proteins containing all three NR boxes. The GRIP-1 construct contains amino acids 629–760 from GRIP-1 and the SRC-1 construct includes amino acids 621–765 from SRC-1.

[b] Amino acids 16–34 from the AR.

with AR but, outside of the LxxLL motif, does not resemble D11 or D30 (Kimbrel, E., and D. P. McDonnell, personal communication).

Several AR mutations have been identified in metastatic prostate cancer which, in addition to being activated by androgens, can also be activated by nonandrogenic compounds. For example, the AR/T877A mutant that contains an alanine at codon 877 instead of a threonine in the wild-type receptor, allows the antagonist OHF to function as an agonist (32, 33). This agonist/antagonist activity switch is considered to be one of the mechanisms by which some prostate tumors escape the inhibitory activity of antiandrogens. Similarly, resistance has been observed after treatment with the antiestrogen tamoxifen in breast cancer patients, where cancer cells switch from recognizing tamoxifen as an ER-antagonist to an agonist (41, 42). In a previous study, we discovered that although the conformation of ER induced by binding to tamoxifen prevents its interaction with p160 coactivators, it enables the presentation of surfaces on the receptor that may be used to recruit other novel coactivators (36, 39). We therefore proposed two scenarios that might explain the agonist/antagonist switch observed in prostate cancers that contains the T877A mutation. One possibility is that, analogous to the mechanism by which tamoxifen resistance arises in breast cancer, the T877A mutation may allow the OHF-bound AR to present a unique surface that permits its interaction with a coactivator with which it would not normally couple. The second possibility is that, in the background of the T877A mutation, OHF-bound AR adopts

a conformation similar to that of the agonist-activated wild-type receptor. To distinguish between these two possibilities, we extended our use of peptide probes to an analysis of the conformation of the AR/T877A mutant in the presence of both DHT and OHF. Surprisingly, we found that OHF-activated AR/T877A interacted with the same peptides as agonist-bound AR (Fig. 1B). This result indicated to us that the agonist/antagonist switch in AR/T877A could be explained by the fact that, in the background of this mutation, the OHF-occupied mutant AR adopts a conformation similar to that of agonist-activated wild-type receptor. Importantly, neither the wild-type nor the T877A mutant is recruited to the D11 and D30 peptides in the presence of bicalutamide, a compound that has been shown previously to function as a pure antagonist for both ARs. These results indicate that the D11 and D30 peptides serve as sensitive probes that detect a receptor conformation that is compatible with transcriptional activation.

D11 and D30 Peptides Interact with the AR-LBD

Previously, we found that all the LxxLL peptides used in this study, including D11 and D30, bind to the coactivator docking surface within the ER-LBD (38). There is, however, evidence suggesting that the LxxLL motifs present in coactivators SRC-1 and GRIP-1 are not important for the docking of these coactivators with AR (18, 43). In addition, our two-hybrid assay also showed that most LxxLL motifs, including those from GRIP-1 and SRC-1, do not interact with AR. We therefore wished to determine if the D11 and D30 peptides function like other coactivator peptides and bind to the region within AR analogous to the coactivator binding pocket in other receptors. Using different fragments of AR in a mammalian two-hybrid assay, we determined that the DBD/LBD (AR507–919) fragment alone was sufficient for D30 (and D11, data not shown) binding (Fig. 2, B and C). In addition, truncation of the amino terminus did not influence peptide binding specificity because peptides that failed to bind the full-length AR

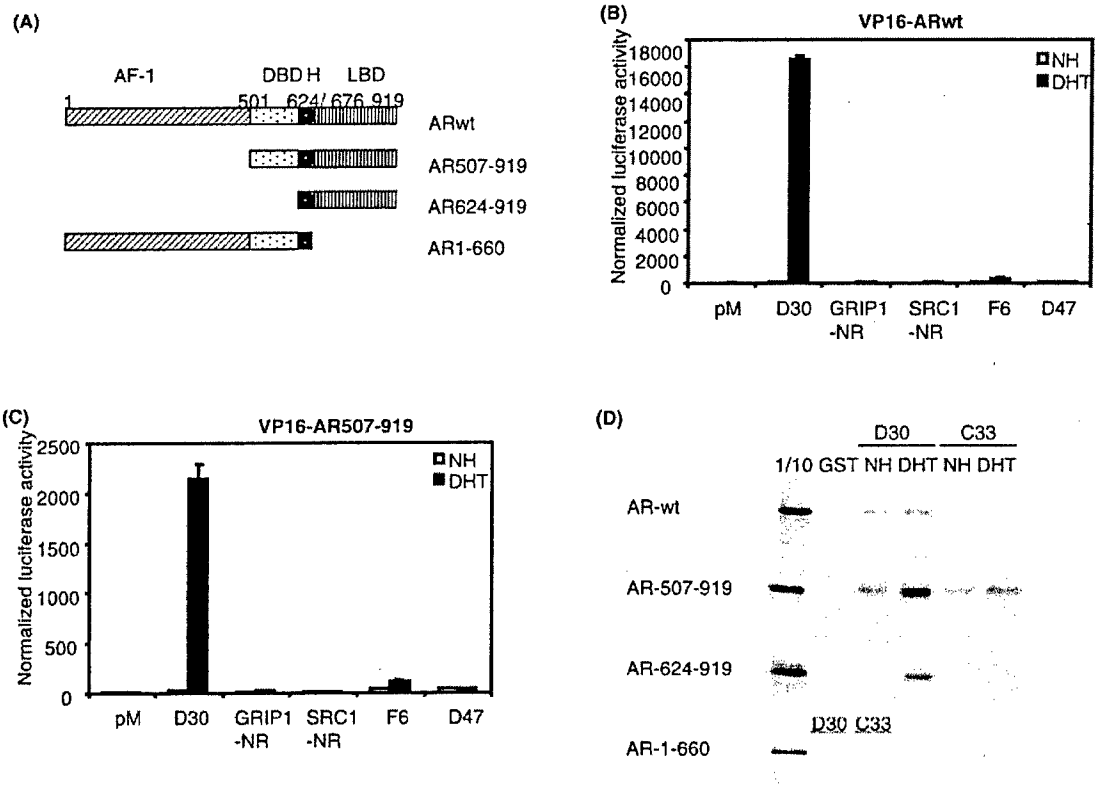

Fig. 2. The D30 Peptide Interacts with the LBD of AR

A schematic diagram of the different AR fragments used is shown in A. CV-1 cells were transfected with different Gal4DBD-peptide fusion constructs and either the VP16-ARwt (B), or the VP16-AR507–919 (C), expression plasmids, together with the 5xGal4Luc3 and pCMV-βgal reporters. After transfection, cells were treated with vehicle control or 100 nM DHT for 16 h and the luciferase and β-galactosidase activities were determined. D, Different fragments of AR were *in vitro* translated and labeled with ^{35}S-methionine. Bacterially expressed GST, GST-D30, and GST-C33 proteins were purified using glutathione-Sepharose beads, and equal amounts of these proteins were incubated with different *in vitro* translated AR fragments in the presence or absence of 1 μM DHT. Nonspecific binding was reduced by four washes with PBST. Proteins remaining bound to the beads were resolved by SDS-PAGE and detected by autoradiography. 1/10, One tenth of the total input protein.

were also unable to bind to the AR DBD-LBD. No interaction with the amino terminus (AR 1–660) was detectable in this assay (data not shown). Similar results were obtained in a GST-pull-down assay. Using the D30 peptide fused to the GST protein, we confirmed that all three constructs, the full length-AR, AR-DBD/LBD, and AR-LBD alone (AR624–919), but not the amino terminus of the receptor (AR1–660), interacted with the D30 peptide (Fig. 2D). Based on these data, we believe that the D30 peptide binds within the LBD of AR.

The D30 Binding Surface Does Not Overlap with the AR: Coactivator Interaction Surface

Because the binding site of the LxxLL peptides overlaps the coactivator docking surface on most of the nuclear receptors, it is not surprising that these peptides, when overexpressed in cells, competitively block coactivator recruitment and inhibit receptor transcriptional activity (38, 40). Because the D30 peptide interacts with AR in an agonist-dependent manner, we wished to determine if this peptide also binds at a site that coincides with or overlaps the coactivator-binding surface on AR. In transfected CV-1 cells, therefore, we overexpressed a two-copy peptide construct, pM-2xD30, to see if it could disrupt AR transcriptional activity. A two-copy peptide was used because we had previously determined that it is more efficient than a single-copy peptide in disrupting ER transcriptional activity (38). Because the D30 peptide also interacts with ER, we were not surprised to see that 2xD30 efficiently inhibited ER transcriptional activity when overexpressed in these cells. We were surprised, however, to find that the 2xD30 only marginally inhibited AR activity when assayed on the MMTV-Luc reporter gene (Fig. 3). Overexpression of this peptide had very little, if any, effect on AR activity when different reporter constructs were used. Similar results were obtained using other cell lines (data not shown). We considered three possible explanations for these results. First, the surface on AR where D30 interacts is not easily accessible when the receptor is DNA bound. Secondly, the D30 binding site on AR does not coincide with a coactivator binding surface; therefore, coactivator binding is not affected. Thirdly, it is possible that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR.

To investigate these possibilities, we first tested whether the conformation of the DNA-bound AR can still be recognized by the D30 peptide. A modified mammalian two-hybrid assay was used in which the expressed AR was allowed to interact with a reporter gene containing an AR response element and the D30 peptide was made so as to contain a VP16 fusion at its amino terminus. Recruitment of the VP16-D30 fusion protein to AR would result in an increase of transcription from the reporter gene. We confirmed with this assay that the D30 peptide is capable of interacting with the DNA-bound AR (data not shown). This result suggests to us that either the D30 binding site on AR does not coincide with a coactivator-binding surface, therefore, coactivator binding is not affected, or that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR.

The D30 Peptide and the Amino Terminus of AR Bind at Overlapping Surfaces on AR-LBD

The findings of several studies from other investigators indicate that the amino and carboxyl termini of AR can interact (7, 18, 44). He *et al.* (45) have also shown recently that an LxxLL-like motif, FxxLF, located at the amino terminus of AR mediates this interaction. Consequently, we considered that the D11 and D30 peptides might be binding to the same surface as this FxxLF motif and therefore used a reconstituted AR transcription assay to address this possibility. In CV-1 cells, the AR DBD-LBD (AR507–919) alone displayed minimal transcriptional activity even when DHT was added to the medium, confirming that the DBD-LBD itself does not recruit coactivators efficiently (Fig. 4A). Because the amino terminus of AR is able to interact

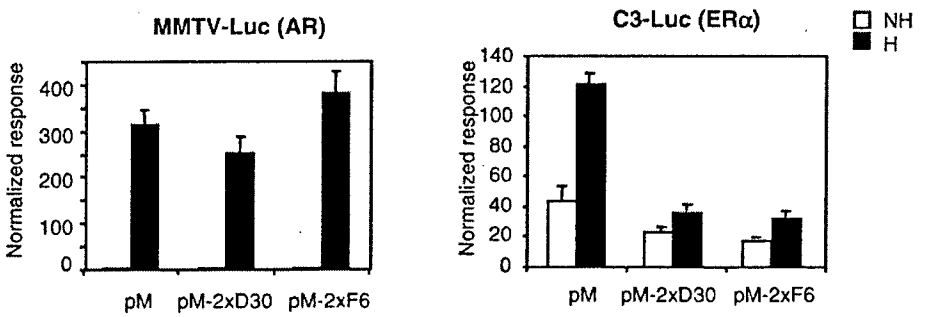

Fig. 3. The D30 Binding Surface on AR Does Not Overlap with that Required for Coactivator Binding

CV-1 cells were transfected with (A) MMTV-Luc, pCMVβgal, and RS-AR or (B) C3-Luc, pCMVβgal, and RST7-ERα in the presence of either pM, pM-2xD30 or pM-2xF6 as indicated. After transfection, cells were treated with either vehicle control or (A) 100 nM DHT or (B) 100 nM E2 for 16 h. Luciferase activity was measured and normalized to the activity of the coexpressed β-galactosidase.

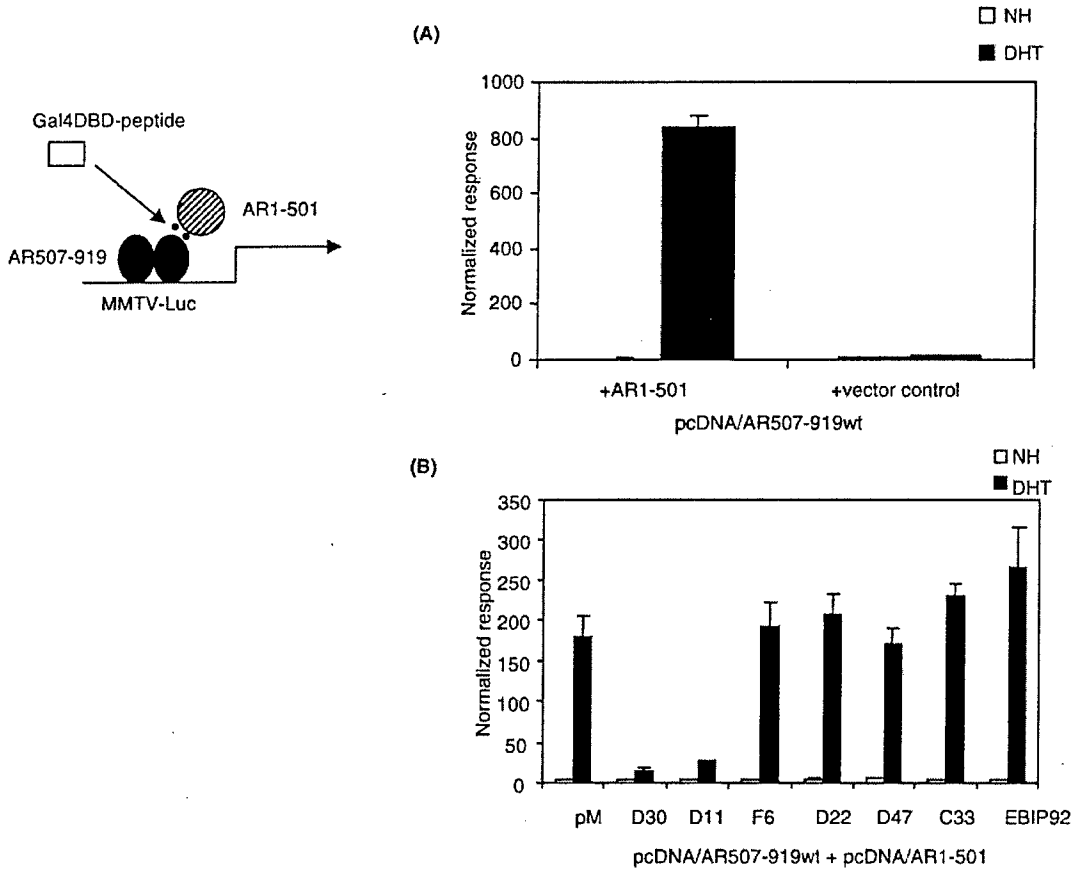

Fig. 4. The D11 and D30 Peptides Bind at a Site in the AR-LBD that Overlaps or Coincides with the Site Where the NH2 Terminus Binds

A, The AR-DBD/LBD alone has minimal transcriptional activity; coexpression of the AR1–501 (N terminus) restores ligand-dependent reporter gene activation. CV-1 cells were transfected with MMTV-Luc, pCMV-βgal, and the AR-DBD/LBD expression plasmid pcDNA/AR507–919wt, together with a control vector or a vector expressing AR1–501. After transfection, cells were treated with vehicle control or 100 nM DHT for 16 h and then assayed for luciferase and β-galactosidase activities. B, The D30 and D11 peptides efficiently blocked the interaction between the amino terminus of AR and the AR-LBD. CV-1 cells were transfected with pcDNA/AR507–919wt, pcDNA/AR1–501, MMTV-Luc, and pCMV-βgal, together with plasmids expressing different Gal4DBD-peptide fusions. Cells were induced and assayed as in A.

with the AR-LBD and is capable of recruiting coactivators on its own, coexpression with the DBD-LBD fragment AR1–501 restores DHT-induced reporter gene activity (Fig. 4A). To test if the D11 and D30 peptides bind to the same surface on AR-LBD as the AR amino terminus, we overexpressed these peptides in the reconstituted system, and found that D11 and D30, but not other LxxLL-containing peptides, could interrupt the AR N/C termini interaction (Fig. 4B). We conclude, therefore, that the D11 and D30 peptides are binding to either the same or overlapping surfaces on LBD where the amino terminus of the receptor binds.

It has been shown in the past that the interaction of the amino terminus of AR with the LBD could stabilize ligand binding to the receptor (46). Because the D30 peptide appears to be binding at the same or overlapping site where the amino terminus of the receptor also binds, we wished to determine if the D30 peptide has the same effect of retaining the ligand in the ligand-binding pocket. This was accomplished by measuring the dissociation rate of a nonmetabolizable ligand, R1881, from AR (Fig. 5). The dissociation half-time (T1/2) of R1881 from full-length AR was approximately 124 min in our assay, and the T1/2 was shortened to about 21 min when the AR507–919 was analyzed in a similar manner. Coexpression of the amino terminus of AR (AR1–501) prolonged the T1/2 to about 47 min and coexpression of the 2xD30 had a similar effect. This result further confirms our theory that the D30 peptide is binding to the same surface on AR-LBD, where the amino terminus of AR also binds. In addition, it suggests that the D30 may serve the same purpose as the amino terminus of the receptor, that of stabilizing the ligand in its binding pocket. This result may also explain why overexpression of the D30 peptide did not inhibit AR transcriptional activity because it functions similarly to the AR N terminus in

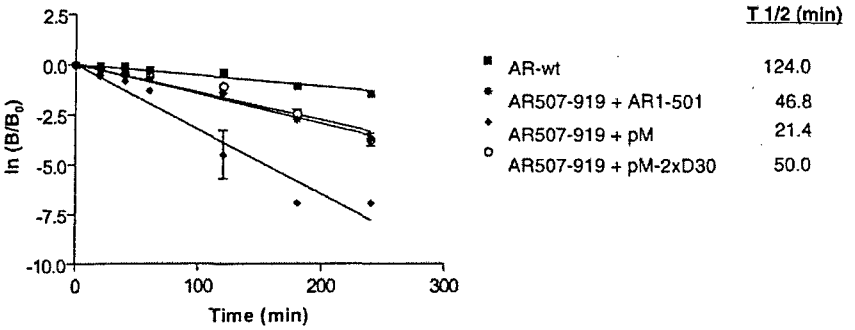

Fig. 5. The D30 Peptide Functions Similarly to the Amino Terminus of AR in Stabilizing Ligand Binding in the AR-LBD

CV-1 cells were transfected with expression plasmids for either 1) wild-type AR alone; 2) AR507–919 plus AR1–501; 3) AR507–919 plus Gal4DBD (pM); or 4) AR507–919 plus Gal4DBD-2xD30 (pM-2xD30). Twenty-four hours after transfection, cells were labeled with 5 nM of ^{3}H-R1881 for 2 h and then a 10,000-fold excess of cold R1881 was added at different time points. Cells were washed four times with PBS to remove nonspecific binding and then lysed for scintillation counting and protein concentration measurement.

stabilizing ligand binding. Although we cannot totally rule out the possibility that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR, our data is most consistent with the hypothesis that the D30/AR interaction resembled more of the AR N/C termini interaction than of cofactor:AR interaction.

Formation of a D30 Docking Site on AR Is Required for Agonist Activity

Although the D30 binding surface within AR is not a coactivator docking site, we were intrigued by the fact that the D30 peptide appeared to recognize the transcriptionally competent AR conformation. We wondered if formation of a D30 binding pocket on AR is required for it to manifest agonist activity and whether the D30:AR interaction indeed predicts AR transcriptional activity. To probe this hypothesis, we compared several agonists and antagonists of AR for their ability to induce reporter gene activation as well as the interaction of AR with D30. The result of this assay is shown in Fig. 6. With MMTV-Luc as a reporter, only DHT was a full agonist for both wild-type AR and the AR/T877A mutant. OHF and RU486 had very little activity when wild-type AR was cotransfected; in contrast, OHF efficiently induced MMTV-Luc expression in the presence of the T877A mutation (Fig. 6A). The ability of these compounds to activate reporter gene expression was compared in parallel with their ability to facilitate the D30:AR interaction (Fig. 6B). The full agonist DHT induced more than a 500-fold interaction between AR and the D30 peptide. RU486, although not as efficient as DHT, induced a 15- to 20-fold interaction between D30 and wild-type AR as well as the T877A AR, which parallels the weak agonist activity of this compound on both ARs. On the other hand, OHF, a potent activator of the T877A mutant but not the wild-type AR, also induced a robust interaction between D30 and VP16-ART877A, but not the wild-type VP16-AR.

Receptor activation, the process of converting AR from an inactive to a transcriptionally active form, requires nuclear translocation, receptor dimerization, DNA binding and recruitment of cofactor proteins. To assess which step requires the formation of a D30 binding pocket, we first tested whether DNA binding can be achieved without the formation of this pocket. We fused both wild-type and T877A AR to VP16 to determine if the ability of compounds to deliver AR to DNA correlates with their ability to induce a D30 binding pocket on AR. This analysis revealed that the wild-type AR activated by OHF was not delivered to DNA efficiently (Fig. 6C). In the presence of the T877A mutation, however, OHF-bound receptor was brought to the DNA as efficiently as that activated by DHT. Interestingly, while RU486 could efficiently deliver both AR-wt and AR/T877A to DNA, the RU486-bound AR was not recruited to the D30 peptide efficiently. This result suggests that the D30 binding pocket is not required for AR to translocate, dimerize, and bind to DNA, but rather is required for events downstream of DNA binding. We have also tested a number of other AR agonists and antagonists (47), and the results confirm that the ability of a compound to induce a D30 binding surface on AR correlates very well with transcriptional activation.

Because we have been able to show that the D30 peptide and the amino terminus of AR compete for binding to the same site on the AR-LBD, we tested whether the AR N-/C-interaction also tracks with transcriptional activation. We found that the AR amino terminus (AR1–501) could be recruited to the AR-DBD/LBD fragment (AR507–919) only when the LBD is in an active conformation, the same conformation that permits the D30 peptide to bind (Fig. 6D). Similar results were also obtained using Gal4DBD-fusions containing the first FxxLF motif in the AR NH2-terminal domain (^{23}FQNLF^{27}) (data not shown). This result suggests that although the AR-LBD is not directly involved in recruiting coactivators, it contributes to shaping a con-

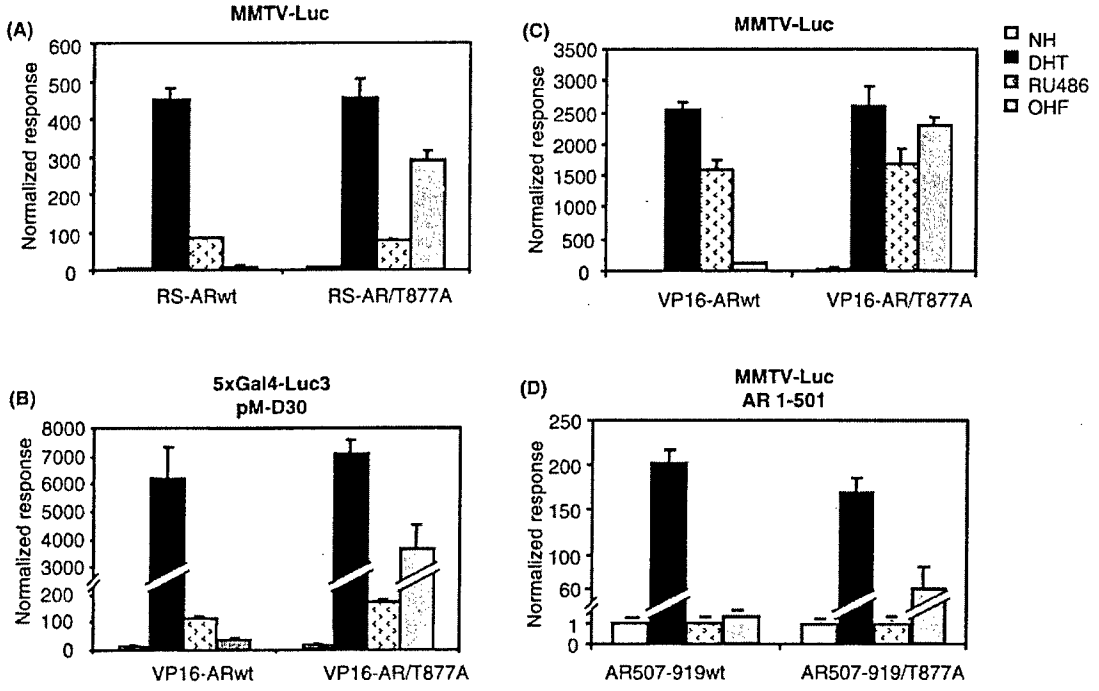

Fig. 6. The Formation of a D30-Binding Pocket on AR Is Required for Its Transcriptional Activity

A reporter gene assay was used to analyze the agonist or antagonist activity of AR ligands. CV-1 cells were transfected with either wild-type or T877A mutant AR expression plasmids, RS-ARwt and RS-AR/T877A, respectively; MMTV-Luc and pCMV-βgal were used as reporter constructs in this assay. B, The mammalian two-hybrid assay was performed to determine the ability of ligand-AR complexes to recruit the D30 peptide. CV-1 cells were transfected with 5xGal4Luc3, pCMV-βgal and pM-30, together with either VP16-ARwt or VP16-AR/T877A. C, The ARwt and AR/T877A were expressed as fusion proteins to the VP16-acidic activation domain to bypass the need for AR-specific coactivators required for gene transcription, allowing the assessment of the ability of ligands to deliver receptors to DNA. CV-1 cells were transfected with pVP16-ARwt or pVP16-AR/T877A together with MMTV-Luc and pCMV-βgal. D, The ability of ligand-AR/LBD complexes to recruit the amino terminus of AR was analyzed. CV-1 cells were transfected with MMTV-Luc, pCMV-βgal and pcDNA-AR1–501, together with either pcDNA-AR507–919wt or pcDNA-AR507–919/T877A. After transfection, cells were treated with either vehicle alone (NH), 100 nM DHT, 100 nM OHF, or 100 nM RU486 as indicated for 16 h before the luciferase and β-galactosidase activities were determined.

formation that is required for AR transcriptional activity. Interestingly, the N/C interaction did not predict the partial agonist activity of RU486 because no N/C interaction was observed in the presence of RU486 (Fig. 6D). This result indicates that the D30/AR interaction may be a more sensitive and/or accurate predictor of AR transcriptional activation.

Several missense mutations have been identified in prostate cancers that appear to increase the agonist efficacy of some androgenic and nonandrogenic ligands. Therefore, we next evaluated whether mutations in the LBD, outside the AF-2 domain, would behave like the T877A mutant and permit the receptor to adopt an active conformation in the presence of a wide variety of ligands. The majority of AR mutations found in prostate cancers are located between codons 670–678, 701–730 (signature sequence), and 874–910 (48). Based on homology modeling, the residues 668–671 do not contribute directly to ligand binding and are positioned away from the ligand binding pocket (49), which suggests that mutations in this region are less likely to influence the binding of the D30 peptide. We therefore decided to focus our analysis on AR mutations found in the latter two regions. The L701H mutant has a reduced sensitivity to the natural ligand DHT; however, it can be activated by physiological concentrations of cortisol (50). As shown in Fig. 7, A and B, the D30 peptide indeed interacted less efficiently with the DHT-activated L701H receptor but gains the ability to interact with this AR variant in the presence of 10 nM cortisol. Another mutation in the signature sequence region, V715M, was shown to have an increased response to progesterone, androsterone, and a number of other endogenous hormones (51, 52). In our analysis, we found that the AR-V715M also recruited the D30 peptide more efficiently in the presence of these ligands (Fig. 7, C and D). In addition to the T877A mutation, another AR variant, H874Y, also contained within the region flanking AF2 (codons 874–910), was found to recognize E2 and hydroxyflutamide as AR agonists (53, 54). In our analysis, we found that the same D30-binding pocket was also formed on the surface of this mutant AR in the presence of these ligands. A full dose-response curve of various ligands used in these analyses (10^{-12}–10^{-6} M) was also performed to ensure that the

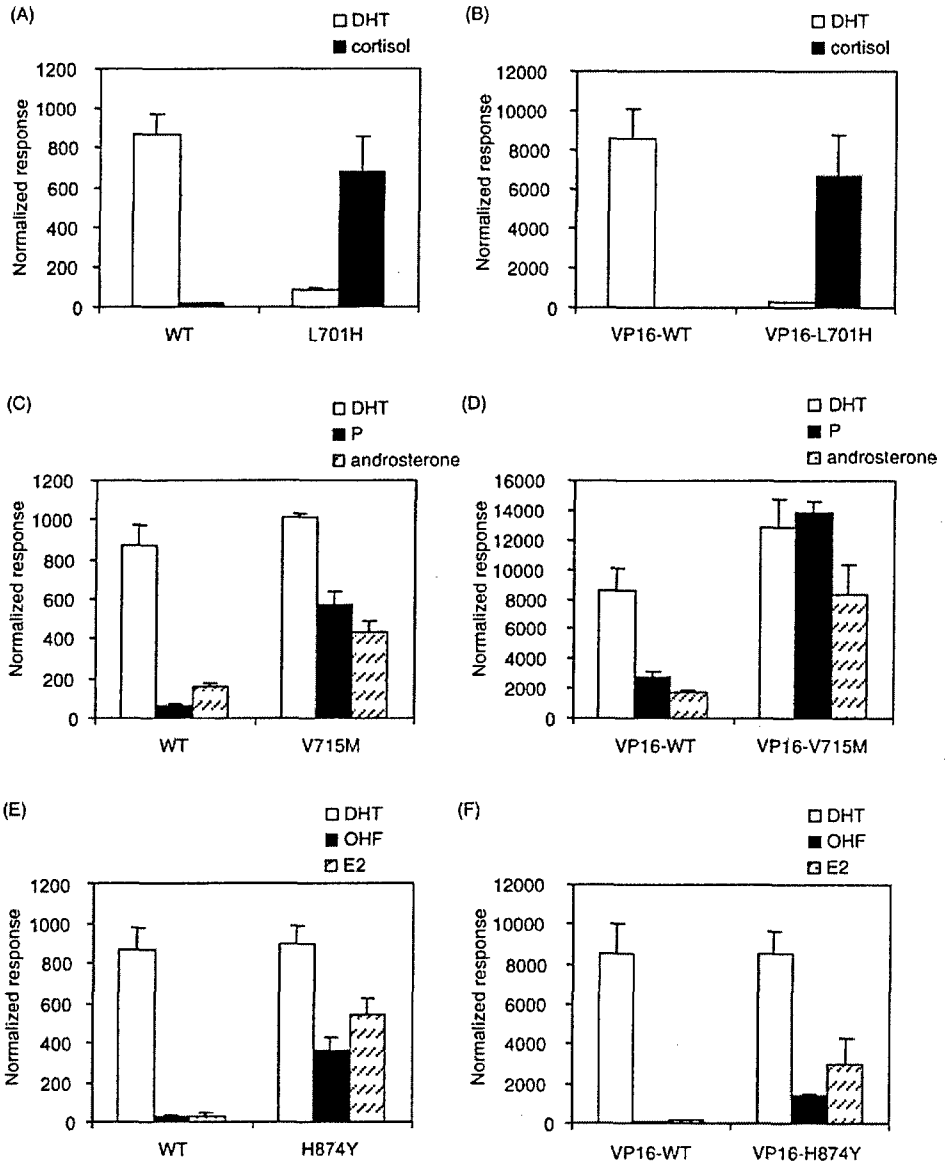

Fig. 7. The D30-Peptide Predicts the Transcriptionally Active Conformation of Various Mutant ARs

A, C, and E, CV-1 cells were transfected with either wild-type or mutant AR expression plasmids, MMTV-Luc and pCMV-βgal. B, D, and F, CV-1 cells were transfected with 5xGal4Luc3, pCMV-βgal and pM-30, together with either VP16-ARwt or VP16-AR mutants. After transfection, cells were treated with either 0.1 nM DHT, 100 nM OHF, 1 nM progesterone (P), 1 nM 17β-estradiol (E2), 10 nM cortisol, or 100 nM androsterone as indicated for 16 h before the luciferase and β-galactosidase activities were determined.

observed phenotype of the cloned mutants reflected that which has been published previously (data not shown). We concluded, therefore, that regardless of the class of mutations, that gain of function phenotype of AR-LBD variants tracks with the acquisition of the ability of AR to adopt an active AF-2 conformation.

DISCUSSION

In this study, we analyzed the ligand-induced structural changes in AR using peptide probes, and found two peptides, D11 and D30, which detect a conformation of AR that is compatible with transcriptional activation. Upon binding an agonist, the LBD of most nuclear receptors adopts an active conformation which allows the presentation of a coactivator binding pocket, permitting the docking of coactivator proteins via a helical LxxLL motif (12–14). We have demonstrated in this report that in the presence of an activating ligand, the AR-LBD undergoes a similar conformational change, allowing the docking of an LxxLL motif contained within the D11 and D30 peptides. While we and others have shown previously that dif-

ferent receptors have distinct LxxLL motif preferences (38, 55), it is noteworthy that AR appears to be one of the most selective, for it interacts with only a very limited subset of LxxLL sequences. Furthermore, although the docking site for these LxxLL peptides in other nuclear receptors is used to recruit coactivators, the analogous region in AR appears to have a different function. Our data agree with previous findings by other investigators that the AR-LBD utilizes this surface to couple with its own amino terminus (44, 45). This interaction slows down the dissociation of ligand from the LBD, maintains the receptor in an active conformation, and thus may allow a more efficient recruitment of coactivators and the subsequent target gene transcription.

Langley *et al.* (44) have shown previously that the OHF binding induced conformational changes within the T877A mutant but not the wild-type AR-LBD, which permitted its interaction with the NH2 terminus of the receptor and that correlated with the activation of this mutant receptor by OHF (44). He *et al.* (45) have also identified an LxxLL-like motif, FxxLF, located within the amino terminus of AR that appears to be responsible for docking the amino terminus of AR to the LBD. Consistent with these data, our results using combinatorial peptide approach have reached the same conclusion and reinforced the hypothesis that the interaction between the amino and carboxyl termini of AR may be required for its transcriptional activity. Our peptide probes appear to have higher sensitivity when compared with the other assays since the partial agonist activity of RU486 is only detected by the D30 peptide but not the N-/C-interaction assays (Fig. 6D). In addition to the ligands examined in this study, we have also tested a large number of other compounds (Ref. 47 and data not shown). All of the results obtained confirm that AR transcriptional activity can be accurately predicted by assaying either the AR N/C termini interaction or the recruitment of VP16-AR to the D30 peptide and that the peptide approach is always the more sensitive of the two assays.

Several factors that appear to contribute to the development of antiestrogen resistance in breast cancers have been identified, including amplification of coactivators, down-regulation of corepressors, and ectopic interactions of ER with cofactor proteins (39, 56–58). We found that the T877A mutation allows AR to adopt an active conformation regardless of whether it is bound by an agonist or antagonist. Because this same active conformation is found in the agonist-bound wild-type AR, it suggests that the consequence of this amino acid change may be to allow mutant AR to appear as a wild-type receptor in target cells. Similarly, the L701H, V715M, and H874Y AR mutants also appeared to function in the same manner by allowing these AR variants to function like wild-type AR in the presence of nonclassical AR ligands. It has been observed that AR mutations occur at a higher frequency in advanced prostate cancers compared with early stage tumors (59–61). All current hormonal manipulations focus on controlling the access of androgens to their cellular receptor, either through elimination of androgens in the circulation or by using an antagonist to compete for their binding to AR. In view of the high frequency of AR mutations identified in advanced cancers, we think that these manipulations will all fail ultimately. A more promising approach would be to develop drugs that target different sites on AR. Based on our theory that the AR amino and carboxyl termini interaction is required for its transcriptional activity, we believe that a peptide or a small molecule that can target the disruption of this interaction would be useful as a therapy for the treatment of antiandrogen refractory prostate tumors. In addition, if the consequence of all AR mutations is to allow the presentation of the same active conformation, it would suggest that both the mutant and wild-type AR may use the same surface to recruit coactivators. Consequently, targeting the interaction between AR and the coactivator(s) required for its transcriptional activity has the potential of being an effective therapy for the treatment of metastatic prostate cancers.

MATERIALS AND METHODS

Reagents and Plasmids

The RS-AR and VP16-AR were gifts from K. Marschke (Ligand Pharmaceuticals, Inc., San Diego, CA) and the plasmids expressing the AR mutants were generated using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA). The 5xGal4Luc3, MMTV-Luc, and pM-peptide plasmids were described previously (38, 62). VP16-AR507–919, VP16-AR624–919, VP16-AR1–660, pcDNA-ARwt, pcDNA-AR507–919, pcDNA-AR624–919, pcDNA-AR1–660, pcDNA-AR1–501 and pM-AR624–919 were generated using PCR amplified AR fragments, and were subcloned into pVP16, pcDNA3 and pM vectors (CLONTECH Laboratories, Inc., Palo Alto, CA, and Invitrogen, Carlsbad, CA). The pVP16–2xD30 and pM-2xD30 plasmids were made in a similar fashion as described before (38), in which two copies of the D30 peptide were fused to the VP16 acidic activation domain and Gal4-DBD, respectively. The GST-D30 plasmid was made by subcloning the D30 peptide into the pGex-6p1 vector (Amersham Pharmacia Biotech, Piscataway, NJ). All the cell culture media and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). CV-1 cells were obtained from ATCC (Manassas, VA). R1881 and ^{3}H-R1881 were obtained from NEN Life Science Products (Boston, MA). DHT was purchased from Sigma (St. Louis, MO). Hydroxyflutamide and bicalutamide were gifts from K. Gaido (CIIT, Research Triangle Park, NC) and Nobex, Inc. (Research Triangle Park, NC).

Cell Culture and Transfection

Monkey kidney CV-1 cells were grown in minimum essential medium plus 8% fetal bovine serum, essential amino acids and sodium pyruvate. Lipofectin-mediated transfection was performed essentially as described (38). Hormones were added after cells recovered from transfection and then all were incubated for 16 h before assaying. Luciferase and β-galactosidase activities were determined as described (38).

GST Pull-Down

Different fragments of AR were *in vitro* translated using pcDNA-ARwt, pcDNA-AR507–919, pcDNA-AR624–919, and pcDNA-AR1–660 as templates and the TNT-rabbit reticulocyte lysate system (Promega Corp., Madison, WI) in the presence of ^{35}S-methionine (Amersham Pharmacia Biotech). GST and GST-D30 proteins were expressed in *Escherichia coli* BL21 cells and purified using glutathione-Sepharose (Amersham Pharmacia Biotech). Sepharose beads containing equal amounts of GST or GST-D30 protein were incubated with *in vitro* translated AR fragments, in the presence or absence of 1 μM DHT overnight at 4 C. The beads were washed 4 times with PBST (PBS containing 0.1% Triton X-100), and proteins remaining bound to the beads were analyzed by SDS-PAGE and visualized with autoradiography.

Measurement of Dissociation Rates

CV-1 cells were seeded in 24-well plates and transfected overnight with different combinations of plasmids as indicated in the figure legend. Transfections were stopped by replacing the transfection mix with phenol-red free medium containing 8% charcoal-stripped FBS, and incubating overnight. For dissociation rate measurement, cells were labeled with 5 nM ^{3}H-R1881 for 2 h, and 10,000-fold excess of cold R1881 was added at different time points. Nonspecific binding was determined from cells that were treated with 5 nM ^{3}H-R1881 in the presence of 10,000-fold excess of cold R1881. The reactions were stopped by washing cells three times with PBS. Cells were dissolved in 200 μl of 1× SDS-buffer (1% SDS; 10 mM Tris, pH 6.8; 10% glycerol), incubated for 10 min at room temperature, and then 300 μl of 10 mM Tris (pH 8.0) was added and the contents were mixed thoroughly by repeat pipetting. For scintillation counting, 400 μl of sample was mixed with 3.5 ml CytoScint (ICN Biochemicals, Inc., Costa Mesa, CA) and counted for 1 min in the Beckman LS-6000 sc scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Protein concentrations were determined by BCA assay (Pierce Chemical Co., Rockford, IL). The specific binding was normalized to the amount of total protein in the sample. The data was plotted and T1/2 calculated using the software Prism 3 (GraphPad Software, Inc., San Diego, CA).

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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 10 Jun 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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